

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
1 February 2001 (01.02.2001)

PCT

(10) International Publication Number
WO 01/07065 A2

- (51) International Patent Classification⁷: **A61K 38/00**
- (21) International Application Number: **PCT/DK00/00425**
- (22) International Filing Date: **27 July 2000 (27.07.2000)**
- (25) Filing Language: **English**
- (26) Publication Language: **English**
- (30) Priority Data:
PA 1999 01071 27 July 1999 (27.07.1999) DK
PA 2000 00667 19 April 2000 (19.04.2000) DK
- (71) Applicant (for all designated States except US): **HEME-BIOTECH A/S [DK/DK]; Roskildevej 12 C, DK-3400 Hillerød (DK).**
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **GELLERFORS, Pär [SE/SE]; Lagmannsvägen 13, S-181 63 Lidingö (SE). FOGH, Jens [SE/DK]; Bjergagervej 37, DK-3540 Lynge (DK).**
- (74) Agent: **PLOUGMANN, VINGTOFT & PARTNERS A/S; Sankt Annæ Plads 11, P.O. Box 3007, DK-1021 Copenhagen K (DK).**
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, DZ, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR (utility model), KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:
— Without international search report and to be republished upon receipt of that report.
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: **PRODUCTION OF rhPBGD AND NEW THERAPEUTIC METHODS FOR TREATING PATIENTS WITH ACUTE INTERMITTENT PORPHYRIA (AIP) AND OTHER PORPHYRIC DISEASES**

WO 01/07065 A2

(57) Abstract: A method for production of rhPBGD in high scale and use of rhPBGD in a method for treatment or prophylaxis of disease caused by deficiency, in a subject, of an enzyme belonging to the heme biosynthetic pathway. The method comprising administering, to the subject, an effective amount of one or more catalyst which is said enzyme or an enzymatically equivalent part or analogue thereof prefeasible in combination with a gene therapy of a mutation related to the catalyst. The disease is selected from the group consisting of acute intermittent porphyria (AIP), ALA deficiency porphyria (ADP), Porphyria cutanea tarda (PCT), Hereditary coproporphyria (HCP), Harderoporphyria (HDP), Variegata porphyria (VP), Congenital erythropoietic porphyria (CEP), Erythropoietic protoporphyria (EPP), and Hepatoerythropoietic porphyria (HEP). The catalyst is one or more enzymes selected from the group consisting of delta-aminolevulinic acid synthetase, delta-aminovulnic acid dehydratase (ALAD), porphobilinogen deaminase (PBGD), uroporphyrinogen III cosynthetase, uroporphyrinogen decarboxylase, coproporphyrinogen oxidase, protoporphyrinogen oxidase, and ferrochelatase, or an enzymatically equivalent part or analogue thereof. In addition the invention relates to the use of rhPBGD. The invention also relates to an expression plasmid pExp1-M2-BB (seq. ID No.1) and to use of a DNA fragment, the EcoR I - Hind III linear fragment (seq. ID No. 2), used for transformation in the hemC disruption strategy for production of rhPBGD expressed in *E. coli*.

PRODUCTION OF rhPBGD AND NEW THERAPEUTIC METHODS FOR TREATING
PATIENTS WITH ACUTE INTERMITTENT PORPHYRIA (AIP) AND OTHER PORPHYRIC
DISEASES

5

FIELD OF THE INVENTION

The present invention relates to novel methods of treating and preventing disease caused by absence or deficiency of the activity of enzymes belonging to the heme biosynthetic
10 pathway. More specifically, the invention pertains to methods of alleviating the symptoms of certain porphyrias, notably acute intermittent porphyria, therapy with a combination of encymatically active substances and therapy with recombinant produced enzymes such as PBGD and ALAD. In addition the invention relates to an expression plasmid and a linear DNA fragment for use in the production of rhPBGD.

15

BACKGROUND OF THE INVENTION

Acute Intermittent Porphyria

20 Acute intermittent porphyria (AIP) is an autosomal dominant disorder in man caused by a defect (50% reduction of activity) of the third enzyme in the heme biosynthetic pathway, porphobilinogen deaminase, (also known as porphobilinogen ammonia-lyase (polymerizing)), E.C. 4.3.1.8. (Waldenström 1937, J. Acta.Med. Scand. Suppl.82). In the following, this enzyme and the recombinant human form will be termed "PBGD" and
25 "rhPBGD", respectively.

Important regulation of the heme biosynthetic pathway is delivered by the end product of the metabolic pathway, namely heme, which exerts a negative inhibition on the first rate-limiting enzymatic step (conducted by delta-aminolevulinic-synthetase) in the heme
30 biosynthetic pathway (Strand et al. 1970, Proc. Natl. Acad. Sci. 67, 1315-1320). Deficiencies in the heme biosynthetic enzymes have been reported leading to a group of diseases collectively called porphyrias. A defect in the third enzymatic step leads to acute intermittent porphyria, AIP. The reduction in enzymatic PBGD activity makes this enzyme the rate limiting step in the heme biosynthetic pathway, with a concomitant increase in
35 urinary and serum levels of delta-aminolevulinic acid (ALA) and porphobilinogen (PBG).

Clinical manifestation of AIP

The clinical manifestation of AIP involves abdominal pain and a variety of neuropsychiatric and circulatory dysfunctions. As a result of the enzymatic block, heme precursors such as PBG and ALA are excreted in excess amounts in the urine and stool. In acute attacks, high levels of PBG and ALA are also found in serum. These precursors are normally undetectable in serum in healthy individuals. The neuropsychiatric disturbances observed in these patients are thought to be due to interference of the precursors with the nervous system or due to the lack of heme. For instance, ALA bears a close resemblance to the inhibitory neurotransmitter 4-aminobutyric acid (GABA) and has been suggested to be a neurotoxin. (Jeans J. et al. 1996, American J. of Medical Genetics. 65, 269-273).

The AIP is a lifelong disease, which usually becomes manifest in puberty. Most precipitating factors exhibit an association with the first rate-limiting enzyme in the heme biosynthetic pathway through heme, the final product of the pathway. A lowering of the heme concentration will immediately increase the rate of ALA-synthetase. An overproduction of ALA then makes the partially deficient PBGD enzyme (50% activity) now rate-limiting with an accumulation of the heme precursors ALA and PBG. Drugs that induces cytochrome P450 such as barbiturates, estrogens, sulphonamides, progesterone, carbamazepine, and phenytoin can all precipitate acute attacks. (Wetterberg L. 1976, In Doss M. Nowrocki P. eds. Porphyrrias in Human Disease. Reports of the discussion. Matgurg an der Lahn, 191-202).

Existing treatment of AIP

25

The treatment of AIP as well as of other types of porphyrias such as variegata, hereditary coproporphyria, harderoporphyria, and aminolevulinic acid dehydratase deficiency, are basically the same. Existing therapies for AIP, are all aimed at reducing circulating PBG and ALA by inhibiting the first rate-limiting enzymatic step ALA-synthetase. This inhibition of ALA-synthetase is achieved by increasing circulating heme, since heme is a negative feed back regulator of ALA-synthetase. Hematin treatment, high caloric intake or inhibition of heme breakdown by Sn-mesoporphyrin administration are the existing therapies today. These therapies have shown limited efficacy.

Levels of ALA and PBG found in urine in patients with symptomatic AIP, are in the range of 1-203 mg/day and 4-782 mg/day, respectively. Normal excretion of ALA and PBG is very low (0-4 mg/day). Important is the observation that these patients also have elevated levels of ALA and PBG in serum. It was shown in a study that AIP patients had significantly
5 elevated levels of ALA (96 μ g %) and PBG (334 μ g %) in serum in connection with acute attacks and that the severity of the attacks were correlated to high levels of ALA and PBG. Hence, it is important to reduce the circulating levels of ALA and PBG in order to eliminate clinical symptoms and to normalize the heme pool.

10 Disclosure of the invention

The present invention is based on using a catalyst such as PBGD, preferably recombinant human PBGD (rhPBGD) and/or ALAD (rhALAD), in order to reduce circulating high levels of PBG in serum by metabolizing (by enzymatic conversion) PBG to hydroxymethylbilane
15 (HMB), which is the normal product of the reaction. This substitution therapy will lead to a normalization of PBG in serum as well as to a normalization of the heme pool. It will also lead to a normalization of ALA in serum, since these heme precursors are in equilibrium with each other. A lowering of serum ALA and PBG is expected to result in a concomitant relief of symptoms. The product of the reaction (HMB) will diffuse back into the cells and
20 enter the normal heme biosynthetic pathway and will become subsequently metabolized to heme.

Hence, PBGD administered by injections will carry out its normal catalytic function by converting PBG to HMB in serum (extracellularly, not inside the cells). The new therapeutic
25 idea is based on the assumption that ALA, PBG and HMB permeate cellular membranes or is transported specifically across them. An alternative to this is to administer a form of PBGD, which will be able to act intracellularly, either as a consequence of formulation or as consequence of modification of PBGD so as to facilitate its entry into cells from the extracellular compartment.

30

The observation that AIP patients have large amounts of these heme precursors in the serum supports the idea that PBG does not accumulate intracellularly, but is released from the cells into serum when the intracellular concentration increases due to the PBGD enzymatic block.

35

By the term "catalyst" is herein meant either the relevant enzyme which is substituted as it is, or an enzymatically equivalent part or analogue thereof. One example of an enzymatically equivalent part of the enzyme could be a domain or subsequence of the enzyme which includes the necessary catalytic site to enable the domain or subsequence to exert substantially the same enzymatic activity as the full-length enzyme or alternatively a gene coding for the catalyst.

An example of an enzymatically equivalent analogue of the enzyme could be a fusion protein which includes the catalytic site of the enzyme in a functional form, but it can also be a homologous variant of the enzyme derived from another species. Also, completely synthetic molecules that mimic the specific enzymatic activity of the relevant enzyme would also constitute "enzymatic equivalent analogues".

The term "the heme biosynthetic pathway" refers to the well-known enzymatic steps (cf. e.g. Sassa S. 1996, Blood Review, 10, 53-58) which leads from glycine and succinyl-CoA to heme, and enzymes belonging to this synthetic pathway are delta-aminolevulinic acid synthetase, delta-aminolevulinic acid dehydratase, porphobilinogen deaminase, uroporphyrinogen III cosynthetase, uroporphyrinogen decarboxylase, coproporphyrinogen oxidase, protoporphyrinogen oxidase and ferrochelatase. Hence, in line with the above, a catalyst used according to the invention is such an enzyme or an enzymatically equivalent part or analogue thereof.

The diseases related to reduced activity of these enzymes are acute intermittent porphyria (AIP), ALA deficiency porphyria (ADP), Porphyria cutanea tarda (PCT), Hereditary coproporphyrria (HCP), Harderoporphyria (HDP), Variegata porphyria (VP), Congenital erythropoietic porphyria (CEP), Erythropoietic protoporphyria (EPP), and Hepatoerythropoietic porphyria (HEP).

By the term "effective amount" is herein meant a dosage of the catalyst which will supplement the lack or deficiency of enzymatic activity in a subject suffering from porphyria caused by reduced activity of one of the above-mentioned enzymes. The precise dosage constituting an effective amount will depend on a number of factors such as serum half-life of the catalyst, specific activity of the catalyst etc. but the skilled person will be able to determine the correct dosage in a given case by means of standard methods (for instance

starting out with experiments in a suitable animal model such as with transgenic animals so as to determine the correlation between blood concentration and enzymatic activity).

The disease which is the preferred target for the inventive method is AIP, and therefore the preferred catalyst is rhPBGD or an enzymatically equivalent part or analogue thereof. The invention further relates to the production of rhPBGD in high scale.

Preferred formulations and dosage forms of the catalyst are exemplified for, but not limited to, PBGD in the detailed description hereinafter, and these formulations also are apparent from the claims. It will be appreciated that these formulations and dosage forms are applicable for all catalysts used according to the invention.

One important aspect of the invention relates to the use of a combination of PBGD and ALAD as well as other combinations of the catalysts disclosed herein.

15

One important embodiment of the method of the inventions is one wherein the catalyst, upon administration, exerts at least part of its enzymatic activity in the intracellular compartment. This can e.g. be achieved when the catalyst is an enzymatically equivalent part or analogue of the enzyme, since such variations of the enzyme can be tailored to render them permeate cell membranes. Hence, when the catalyst is a small artificial enzyme or an organic catalyst which can polymerize porphobilinogen to hydroxymethylbilane, it should be possible for the skilled man to introduce relevant side chains which facilitates entry into the intracellular compartment. Alternatively, the catalyst is the enzyme, but formulated in such a manner that it exerts at least part of its enzymatic activity intracellularly upon administration to the subject. This can be achieved by tagging the enzyme with specific carbohydrates or other liver cell specific structures for specific liver uptake, i.e. the enzyme (or analogue) is modified so as to facilitate active transport into e.g. liver cells.

Although the above embodiments are interesting, it is believed that the normal, practical embodiment of the invention will involve use of a catalyst which exerts substantially all its enzymatic activity extracellularly in the bloodstream, since it is believed that the metabolic products of the enzymatic conversion of the relevant heme precursor will permeate freely into the intracellular compartment where the remaining conversions of the heme

biosynthetic pathway can take place. Alternatively, the metabolic product may be excreted from the subject via urine and/or faeces at least to some extent.

The method for recombinant production comprises

5

a) introducing, into a suitable vector, a nucleic acid fragment which includes a nucleic acid sequence encoding the catalyst;

b) transforming a compatible host cell with the vector;

10 c) culturing the transformed host cell under conditions facilitating expression of the nucleic acid sequence; and

d) recovering the expression product from the culture

and optionally subjecting the expression product to post-translational processing, such as in vitro protein refolding, enzymatic removal of fusion partners, alkylation of amino acid residues, and deglycosylation, so as to obtain the catalyst.

15

For relatively small catalysts (e.g. those constituted mainly of the active site of the enzyme), the catalyst can alternatively be prepared by liquid-phase or solid-phase peptide synthesis.

20 A more detailed explanation of the recombinant production of the model enzyme PBGD is given in the detailed section hereinafter, but as mentioned herein the same considerations apply for all other peptide catalysts of the invention. One of the main advantages of producing the catalyst by recombinant or synthetic means is, that if produced in a non-human cell, the catalyst is free from any other biological material of human origin, thus
25 reducing problems with known or unknown pathogens such as viruses etc.

The dosage regimen will normally be comprised of at least one daily dose of the catalyst, (preferably by the i.v., s.c. i.p., trans dermal or trans mucosal route including nasal and buccal administration). Normally 2, 3, 4 or 5 daily dosages will be necessary, but if
30 sustained release compositions or e.g. sub cutane injections are employed, 1 or less than 1 daily dosage are anticipated to be sufficient.

The daily dosage should be determined on a case by case basis by the skilled practitioner, but as a general rule, the daily dosage will be in the range between 0.01 – 1.0 mg/kg body
35 weight per day of the catalyst. More often the dosage will be in the range of 0.05 – 0.5

mg/kg body weight per day, but it should never be forgotten that precise dosage depends on the dosage form and on the activity of the catalyst as well as on the degree of deficiency of the relevant enzyme or combinations of enzymes and an individualized treatment, where the dose is adjusted to normalize patient serum and urine precursor levels.

5

The most correct way of determining the correct dosage is based on the patient specific precursor levels. The precursor being the product of the enzymatic reaction.

For PBGD, the daily dosage is about 0.08-0.2 mg per kg body weight per day, and most often 0.1 mg per kg body weight per day will be the dosage of choice. It is believed that comparable dosages will be applicable for the other full-length enzymes or combinations of enzymes.

The invention also pertains to a catalyst as defined herein for use as a pharmaceutical.

15 Furthermore, use of such catalysts or combination of different catalysts for the preparation of pharmaceutical compositions for treatment of the above-discussed diseases is also part of the invention.

Legends to figures:

20

Figure 1: Circular map of plasmid pPBGD1.1

Figure 2: Flow chart for construction of plasmid pExp0

Figure 3: Circular map of plasmid pExp0

Figure 4: Flow chart for construction of plasmid pExp1

25 Figure 5: Circular map of plasmid pExp1

Figure 6: Flow chart for construction of pExp1-M2

Figure 7: Circular map of plasmid pExp1-M2

Figure 8: Flow chart for construction of rhPBGD expression plasmid pExp1-M2-BB

Figure 9: Circular map of rhPBGD expression plasmid pExp1-M2-BB

30 Figure 10: PCR strategy for construction of the *EcoR* I-*Hind* III linear DNA-fragment

Figure 11: Structure of the *EcoR* I-*Hind* III linear DNA-fragment used for transformation

Figure 12: Respiration and growth data from fermentation PD14 with strain PBGD-2

Figure 13: rhPBGD expression in fermentation PD14 with strain PBGD-2

Figure 14: Chromatography on DEAE-Sepharose FF (DEAE1)

35 Figure 15: Chromatography on DEAE-Sepharose FF (DEAE2)

- Figure 16: Chromatography on Butyl-Sepharose 4 FF
- Figure 17: Circular map of rhPBGD-His expression plasmid pExp2
- Figure 18: PBGD reaction mechanism
- Figure 19: DEAE chromatography elution profile
- 5 Figure 20: SDS-PAGE gel of DEAE eluates
- Figure 21: Cobalt chromatography elution profile
- Figure 22: SDS-PAGE gel results of cobalt eluates
- Figure 23 and
- Figure 24: Illustrate numbers in diagrams (Table 19). The expression of PBGD in HeLa cells
- 10 was increased up to 475 times from the basal activity and in NIH 3T3 cells up to 11 times.
- Figure 25: Comparison of fermentations PD05 and PD06 with strain PBGD-2
- Figure 26: Comparison of fermentations PD09, PD11 and PD12
- Figure 27: Comparison of fermentations PD09, PD11 and PD12 with strain PBGD-1.
- Figure 28: Comparison of fermentations PD14, PD16 and PD19 with strain PBGD-2.
- 15
- Figure 29: Comparison of fermentations PD14, PD16 and PD19 with strain PBGD-2
- Figure 30: Comparison of fermentations PD19, PD21 and PD22 with strain PBGD-2.
- Figure 31: Comparison of fermentations PD19, PD21 and PD22 with strain PBGD-2.
- Figure 32: Comparison of fermentations PD19, PD1501 and PD1502
- 20 Figure 33: Comparison of fermentations PD19, PD1501 and PD1502 with strain PBGD-2.
- Figure 34:
- Figure 35: Stability studies: Single use aliquots of extract were routinely taken out of the freezer (-20°C) and the rhPBGD-activity was measured and plotted over time.
- Figure 36: Description of oligos used for PCR amplification.
- 25 Figure 37 A, and B: Strategy for PCR cloning of ALAD
- Figure 38: Plasma levels of rhPBGD following administration to mice. 50 µg rhPBGD (2,3-2,8 mg/kg)
- Figure 39: PBGD enzymatic activity in plasma following rhPBGD administration to mice
- Figure 40: The urinary content of PBG and ALA in AIP-mouse treated with phenobarbital.
- 30 Figure 41 shows the urinary content of PBG and ALA in AIP-mouse treated with phenobarbital and rhPBGD.
- Figure 42: Shows the grip strength analysis in control and AIP-mice
- Figure 43: Rotarod analysis in control and AIP-mice. The rotarod analysis were determined using a rotarod treadmill (Ugo Basile) in wild type controls (control, n=5) and in AIP-
- 35 transgenic mice (AIP, n=7).

Figure 44. Enzyme concentration over 8 weeks at 40°C measured by HPLC. A decrease from 2 mg/ml to 0,5 mg/ml and 8 mg/ml to 2,5 was detected.

Figure 46. Enzyme specific activity measured during 8 weeks at 40°C. The activity was measured using the enzyme activity assay and the protein concentration was measured
5 using HPLC.

Figure 45. The enzyme activity measured over 8 weeks at 40°C. A significant decrease over the first week was seen for the high concentration sample, 1b. After two weeks the decrease rate was the same for all samples.

Figure 47. rhPBGD concentration over 12 weeks at -20°C (freezer), 5°C (fridge), 25°C
10 (RT) and freeze/thawed at each sampling. The measurement was performed using HPLC
Figure 48. rhPBGD activity over 12 weeks at -20°C (freezer), 5°C (fridge), 25°C (RT) and freeze/thawed at each sampling.

Figure 49. rhPBGD specific activity over 12 weeks at -20°C (freezer), 5°C (fridge), 25°C
(RT) and freeze/thawed at each sampling. Measurements were performed using enzyme
15 activity assay and HPLC.

Figure 51. The rhPBGD activity measured over 8 weeks. The stability study has been performed under nitrogen at -20°C ± 5°C, 5°C ± 3°C and at 25°C ± 2°C.

Figure 51. The rhPBGD activity measured over 8 weeks. The stability study has been performed under nitrogen at -20°C ± 5°C, 5°C ± 3°C and at 25°C ± 2°C.

20 Figure 52. The specific rhPBGD activity measured using the enzyme activity assay and BCA protein concentration assay. The stability study has been performed under nitrogen at -20°C ± 5°C, 5°C ± 3°C and at 25°C ± 2°C.

Sequence list:

25

Seq. ID NO 1: Sequence of the expression plasmid pExp1-M2-BB

Seq. ID NO 2: Sequence of the EcoR I - Hind III linear fragment used for transformation in the hemC disruption strategy

Seq. ID NO 3: Sequence of the erythropoietic form (PBGD 1.1)

30 Seq. ID NO 4: Sequence of the non-erythropoietic form (PBGD 1.1.1)

Seq. ID NO 5: Sequence of PDGB from Spleen (PBGD 1.3)

Seq. ID NO 6: Sequence of PDGB from bone marrow (PBGD 2.1)

Seq. ID NO 7: Sequence of PDGB from bone marrow (PBGD 2.2)

Seq. ID NO 8: Sequence of PDGB from lymph node (PBGD 3.1)

35 Seq. ID NO 9: Sequence of PDGB from lymph node (PBGD 3.3)

- Seq. ID NO 10: Sequence PDGB from total brain (PBGD 5.3)
Seq. ID NO 11: Sequence of PDGB from total brain (PBGD 6.1)
Seq. ID NO 12: Sequence of PDGB, Fig 1
Seq. ID NO 13: Sequence of (PBGD)
5 Seq. ID NO 14: Primer named ICO 549
Seq. ID NO 15: Primer named ICO 550
Seq. ID NO 16: Primer named ICO 383
Seq. ID NO 17: Primer named ICO 384
Seq. ID NO 18: Primer named ICO 618
10 Seq. ID NO 19: Primer named ICO 616
Seq. ID NO 20: Primer named ICO 617
Seq. ID NO 21: Sequence of ALAD coding region of pBlueAalD-2
Seq. ID NO 21: Proteinequence of ALAD coding region of pBlueAalD-2

15

DETAILED DISCLOSURE OF THE INVENTION

- In a first embodiment the invention relates to a method for treatment or prophylaxis of disease caused by deficiency, in a subject, of one or more enzymes belonging to the heme biosynthetic pathway, the method comprising administering, to the subject, an effective amount of a catalysts which is said enzyme or combination of enzymes or an enzymatically equivalent part or analogue thereof. The disease may be selected from the porphyria group and the catalyst may be an enzyme selected from the group consisting of
- 20 delta-aminolevulinic acid synthetase,
25 delta-aminolevulinic acid dehydratase (ALAD),
porphobilinogen deaminase (PBGD),
uroporphyrinogen III cosynthetase,
uroporphyrinogen decarboxylase,
coproporphyrinogen oxidase,
30 protoporphyrinogen oxidase, and
ferrochelatase,
or an enzymatically equivalent part or analogue thereof.

- The invention also relates to any combination of the enzymes mentioned above because
- 35 one enzymatic deficiency may cause such alterations of the pathway that alternative

enzymatic reactions are needed wherein an otherwise normal production of an enzyme for such alternative pathway is not sufficient. In alternative, the disease relating to the heme biosynthetic pathway may also be due to a deficiency of more than only one enzyme. Accordingly, in the present context the term catalyst is also to be interpreted as a
5 combination of catalyst and the term enzyme may also include a mixture of different enzymes.

In a preferred embodiment, the disease is AIP and the enzyme is PBGD or an enzymatically equivalent part or analogue thereof optionally in combination with ALAD. In a
10 further embodiment, the catalyst is a recombinant form of the enzyme belonging to the heme biosynthetic pathway or of the enzymatically equivalent part or analogue thereof.

The catalyst may be administered by a route selected from the group consisting of the intravenous route, the intraarterial route, the intracutaneous route, the subcutaneous route,
15 the oral route, the buccal route, the intramuscular route, the anal route, the transdermal route, the intradermal route, and the intratechal route.

The catalyst is preferable formulated in an isotonic solution, such as 0.9% NaCl and 10-50 mM sodium phosphate pH 7.0 +/- 0.5 up to pH 8.0 or sodium phosphate, glycine, mannitol
20 or the corresponding potassium salts. The catalyst may also be lyophilized, sterile filtered, and in a further embodiment formulated as lipid vesicles comprising phosphatidylcholine or phosphatidylethanolamine or combinations thereof. In a still other embodiment the catalyst is incorporated into erythrocyte ghosts.

25 Also a sustained release formulation may be performed involving biodegradable microspheres, such as microspheres comprising polylactic acid, polyglycolic acid or mixtures of these.

A further method according to the invention is wherein the catalyst is lyophilized in a two-
30 compartment cartridge, where the catalyst will be in the front compartment and water for reconstitution in the rear compartment. The two compartment cartridge may be combined with an injection device to administer the catalyst either by a needle or by a needle-less (high pressure) device.

It may also be very convenient to administer the catalyst in a formulation of a physiological buffer containing an enhancer for nasal administration.

Other formulations for the catalyst include an oral formulation containing lipid vesicles,
5 such as those comprising phosphatidylcholine, phosphatidylethanolamine, or sphingomyeline, or dextrane microspheres.

The formulation is preferable one that is able to enhance the half-life of the catalyst in the subject's bloodstream. This may be use of a formulation wherein the catalyst has a
10 polyethylene glycol coating.

The catalyst may also be complexed with a heavy metal.

In a further aspect the catalyst is an enzymatically equivalent part or analogue of the
15 enzyme and exerts at least part of its enzymatic activity intracellularly upon administration to the subject. This may be when the catalyst is a small artificial enzyme or an organic catalyst that can polymerise porphobilinogen to hydroxymethylbilane.

Furthermore, the catalyst may be said enzyme formulated in such a manner that it exerts at
20 least part of its enzymatic activity intracellularly upon administration to the subject.

In addition the catalyst may be tagged with specific carbohydrates or other liver cell specific structures for specific liver uptake.

25 In a further aspect the catalyst exerts substantially all its enzymatic activity extracellularly in the bloodstream.

In a still further aspect, the enzymatic activity of the catalyst on its relevant heme precursor results in a metabolic product which 1) either moves into the intracellular compartment and
30 is converted further via the remaining steps of the heme biosynthetic pathway or 2) is excreted from the subject via urine and/or faeces.

A primary embodiment of the invention relates to a method wherein the catalyst has been prepared by a method comprising

35

- a) introducing, into a suitable vector, a nucleic acid fragment which includes a nucleic acid sequence encoding the catalyst;
b) transforming a compatible host cell with the vector;
c) culturing the transformed host cell under conditions facilitating expression of the nucleic acid sequence; and
d) recovering the expression product from the culture
and optionally subjecting the expression product to post-translational processing, such as in vitro protein refolding, enzymatic removal of fusion partners, alkylation of amino acid residues, and deglycosylation, so as to obtain the catalyst.

10

A further catalyst in a combination may be prepared by liquid-phase or solid-phase peptide synthesis and it is preferable free from any other biological material of human origin.

As mentioned above the catalyst may be administered at least once a day, such as 2, 3, 4, and 5 times daily depending on the specific treatment regimen outlined for the patient in that precursor levels for each patient are measured before and/or during treatment for evaluation of the specific dosage.

Accordingly the daily dosage may be in the range of 0.01 – 1.0 mg/kg body weight per day, such as in the range of 0.05 – 0.5 mg/kg body weight per day. And the present invention also relates to the use of the catalyst for the preparation of a pharmaceutical composition.

It is estimated that a dosage will often be about 0.1 mg per kg body weight per day.

Accordingly, the invention also relates to a catalyst which is an enzyme of the heme biosynthetic pathway or an enzymatically equivalent part or analogue thereof, for use as a medicament. Thus in a further embodiment, the invention relates to a catalyst which is an enzyme of the heme biosynthetic pathway or an enzymatically equivalent part or analogue thereof for the preparation of a pharmaceutical composition for the treatment or prophylaxis of diseases caused by deficiency of said enzyme.

Naturally, the catalyst may be a recombinant form of the enzyme. An example is a recombinant human PBGD based on any of Seq. ID NO 3 and Seq. ID NO 4.

In a preferred embodiment and as will be disclosed in detail below, the invention also relates to a method for treating a patient having one or more mutations in the PBGD gene causing an enzyme defect, the method comprising use of a human PBGD cDNA sequence of either non-erythropoietic form or erythropoietic form according to the tissue in which PBGD
5 should be expressed, and transfecting the patient with the relevant cDNA. Preferably the enzyme deficiency is selected from enzyme deficiencies resulting in a disease selected from Acute intermittent porphyria, (AIP), ALA deficiency porphyria (ADP), Porphyria cutanea tarda (PCT), Hereditary coproporphyria (HCP), Harderoporphyria (HDP), Variegata porphyria (VP), Congenital erythropoietic porphyria (CEP), Erythropoietic
10 protoporphyria (EPP), and Hepatoerythropoietic porphyria (HEP). In a still more preferred embodiment, the gene therapy is combined with administration of a recombinant enzyme according to the present invention.

In a preferred embodiment, the human PBGD cDNA sequence is selected from Seq. ID NO
15 3 and Seq. ID NO 4.

The transfection may be by use of a vector selected from adenovirus, retrovirus and associated adenovirus. The PBGD gene transfer vector into human cells (erythropoietic and/or non-erythropoietic) preferable results in normal PBGD activity or in an activity
20 wherein the patient is free of symptoms of disease.

A further method of gene therapy treatment of patients with Acute Intermittent Porphyria (AIP) is by a correction of one of the specific point mutations identified causing AIP by use of chimeraplasty gene repair. This involves specific designed oligonucleotides and a specific
25 knowledge of both the mutation to be corrected and to the sequence on both sides on the mutation. In a specific embodiment of chimeraplasty gene repair is by use of a delivery system for transfection by use of non-viral vectors formulated in a vehicle preparation comprising one or more components selected from cationic phospholipids, phospholipids, phospholipids mixed with neutral lipids, lictosylated PEI, liposomes liposomes comprising
30 mixtures of natural phospholipids and neutral lipids.

The mutation may be selected from the mutations shown in Table A.

The following description of preferred embodiments of the invention will focus on
35 recombinant production of PBGD and formulations and uses thereof. It will be appreciated,

however, that all disclosures relating to this polypeptide apply also for the other enzymes mentioned above. Hence, production and use of PBGD only exemplifies the invention, but all other enzymes of the heme biosynthetic pathway can substitute PBGD in the embodiments described hereinafter.

5

Production of recombinant human PBGD (rhPBGD)

As mentioned above, it is preferred to administer recombinant human versions of the various enzymes of the heme biosynthetic pathway. In the following will be described
10 recombinant production of one of these enzymes, namely PBGD.

The gene for the erythropoietic PBGD, which is located in the human genome in the chromosomal region 11q 24, is composed of 15 exons spanning 10 kb of DNA and is shown in Grandchamp B. et al. 1996, J. of Gastroenerology and Hepatology 11, 1046-
15 1052.

The gene coding the erythropoietic PBGD enzyme (344 amino acids) (Raich N. et al 1986, Nucleic. Acid. Res, 14, 5955-5968), will be cloned from a human erythropoietic tissue by use of a nested PCR (polymerase chain reaction) strategy.

20

The PBGD coding region will be inserted in a plasmid and transformed into a suitable host cell (a bacterium such as *E. coli* and *B. subtilis*, or a fungus such as *Saccharomyces*). The expression of the PBGD gene will be regulated by a promoter which is compatible with the selected host cell.

25

For bacterial production: An endogenous ATG sequence is located at the NH₂-terminal end of the PBGD structural gene for initiation of translation and cytoplasmic expression. Alternatively insert in front of the PBGD coding region a bacterial signal sequence for example an *E. coli* periplasmic enzyme signal peptide or a signal peptide from a secreted
30 enterotoxin or endotoxin in *E. coli*, to obtain secretion in *E. coli*.

A plasmid used for production of rhPBGD in *E. coli* was constructed in the following way:

Construction of a plasmid harboring the coding region of human wild type PBGD

35 (pBPBGD1.1)

Introduction:

The erythropoietic expressed form of porphobilinogen deaminase (PBGD) (Raich N. et al. 5 Nucleic Acids Research 1986 14(15): 5955-67) was cloned and sequence determined. Two forms of PBGD are known. The erythropoietic form is expressed specifically in erythroid progenitors and the constitutive form is expressed in all cells (Grandchamp B. et al. 1987, Eur J Biochem. 162(1):105-10). The two are expressed from the same gene and are identical except for the addition of 17 amino acids at the amino terminus of the constitutive 10 form through alternative exon usage. It was decided to clone and express the erythropoietic form. There are three sequences for PBGD in the Genbank, the two isoforms mentioned above and the genomic sequence (Yoo HW. et al. 1993, Genomics. 15(1):21-9). These all have nucleotide differences translating to amino acid changes. Before choosing a specific sequence to be expressed for a human therapeutic it was 15 therefore necessary to determine what is the wild type allele. To accomplish this, PBGD cDNA clones were isolated and sequenced from a number of sources to define the most common amino acid usage. Oligonucleotide primers were designed to amplify the coding region from cDNAs by Polymerase Chain Reaction (PCR) (Saiki R.K. et al. 1985, Science 230(4732):1350-4). These were used to isolate cDNAs from 5 sources of mRNA which 20 were then cloned into a plasmid vector. Eight of these clones were sequenced and along with the published sequences define a wild type allele, which should be the most common amino acid sequence in the population. This wild type allele will be used for protein expression.

25 Strategy:

A nested PCR strategy was devised to clone PBGD. The first primer set, (see Table 1) Ico379 and Ico382, are 20mers that bind to sequence outside of the coding region. Ico379 is specific for the 5' untranslated region of the mRNA (cDNA) of the erythropoietic form of 30 PBGD. The binding site is in an exon region not expressed in the constitutive form of the enzyme. Ico382 binds to the 3' untranslated region of both forms of PBGD. Internal to these are a second set of oligonucleotide primers to be used for the second round of PCR, Ico375 and Ico376, designed to distal ends of the PBGD coding region. Ico375 has 22 bases of sequence homologous to the 5' end of the coding region of the erythropoietic form 35 of PBGD with the ATG start codon followed by an *EcoR* I endonuclease cleavage site for

cloning of the PCR product and 4 bases of sequence to ensure efficient restriction. Ico376 has 33 bases homologous to the 3' end of the PBGD coding region with 3 bases changed internally to introduce a *Mun I/Mfe I* endonuclease cleavage site through silent mutations and ending with the TAA stop codon. This restriction site will be used to easily introduce
5 sequence encoding a His-Tag to the DNA with oligonucleotide adapters or to enable other 3' modifications. Following the stop codon is a second stop codon to ensure good termination of translation and a *Hind III* endonuclease cleavage site for cloning the PCR product followed by 4 bases to ensure efficient restriction. The *EcoR I* and *Hind III* endonuclease cleavage sites introduced onto the ends of the PBGD PCR product ligate
10 into the respective unique restriction sites in the high copy number pBluescriptII SK- (Stratagene) vector for sequencing and will then be used to move the PBGD DNA into an *E. coli* expression vector for production of recombinant human porphobilinogen deaminase, rhPBGD.

15 PCR:

Six cDNAs were used as a PCR source; spleen, bone marrow, lymph node, lung, whole brain and adipose tissue each from a different pool of human donors (produced by Donald Rao using BRL Superscript II with 500 ng Clontech poly-A RNA in 20 µl reaction volumes
20 per manufacturers instructions except adipose which was made from 5 µg of Clontech total RNA from a single donor). List of equipment and supplies used (see lists below). One µl of each cDNA (approximately 25ng) was amplified with Advantage cDNA polymerase mix (Clontech) with 0.2mM dNTP (PE/ABI) and 0.3 µM each of Ico379 and Ico382 in 50 µl reaction volumes. Two cycle PCR was used, with an initial heat denaturation step at 94°C
25 for 1' 40" then 28 cycles of 96°C for 16" and 68°C for 2'. A final extension of 6' at 74°C ensured that extension products were filled out. One fifth of the reaction was run out on a 1.2% agarose gel with 2 µl of 6X ficol loading dye in 0.5X TBE buffer (Maniatis T., E.F. Fritsch, J. Sambrook. Molecular Cloning (A laboratory Manual) Cold Spring Harbor Laboratory. 1982). The predicted band of 1.1 kb. was observed by ethidium bromide
30 staining with all sources but lung tissue cDNA. These bands were excised and DNA was isolated with Microcon-30 with micropure inserts (Amicon/Millipore) per manufacturers instructions and buffer exchanged with dH₂O. One tenth of the recovered DNA was amplified with Advantage cDNA polymerase mix (Clontech) with 0.2mM dNTP (PE/ABI) and 0.3µM each of the internal nested oligonucleotides (Ico375 and Ico376) at 0.3 µM in
35 50µl reactions. Two cycle PCR was used again with an initial heat denaturation step at

94°C for 1' 40" then 2 cycles of 96°C for 16" and 68°C for 2' then 13 cycles of 96°C for 16" and 72°C for 2' with a final extension of 6' at 74°C. Ten µl of the 50 µl reactions were run on a 1.2% agarose gel with 2 µl 6X loading dye. The resulting bands were of the expected size, 1.05 kb. The remainder of the PCR reactions were passed through Chromaspin-400
5 columns (Clontech) per manufacturers instructions to remove reaction components and to exchange buffer with TE (10mM Tris-HCl pH8.0/ 1mM EDTA). The DNA containing eluates were washed with dH₂O and concentrated with Microcon-100 spin-filters (Amicon/Millipore) as described by the manufacturer's instructions.

10 Cloning:

The purified PBGD DNA was digested for 6 hours with 40 Units each of *EcoR* I and *Hind* III in *EcoR* I "U" buffer (New England Biolabs (NEB)) in 50 µl reactions at 37°C. Enzymes were heat killed for 20 minutes at 68°C and reactions were spun in Microcon 100 spin-
15 filters to remove small DNA end pieces, washed with dH₂O and concentrated. One fifth of the resulting DNA was ligated with approximately 50 ng *EcoR* I and *Hind* III digested and twice gel purified pBluescriptII SK- (Stratagene) and 200 units T4 DNA ligase (NEB cohesive end units) for 15 hours at 16°C. The ligase was heat killed at 75°C for 10 minutes. The reactions were then buffer exchanged with dH₂O and concentrated in Microcon-100
20 spin filters and volumes taken up to 5 µl with dH₂O. One µl each was electroporated into 25 µl DH10B Electromax cells (Gibco/BRL) at 2.5Kv/200Ohms/25µF in 0.1cm cuvetts with a BioRad electroporator. One ml of SOC medium (Gibco/BRL) was added and the cells were outgrown at 37°C for one hour at 250 rpm. Cells were plated out on LB plates (Maniatis T., E.F. Fritsch, J. Sambrook. Molecular Cloning (A laboratory Manual) Cold Spring Harbor
25 Laboratory. 1982) with 150 µg/ml ampicillin. The efficiency of all five were approximately twice background (vector ligated without insert). Colony PCR was used to analyze 18 transformants of each electroporation for the presence of PBGD. An internal PBGD specific primer (ICO381) was used with a pBluescript specific primer (ICO385) to both confirm identity and proper orientation in the vector. The 25 µl reactions were set up on ice to
30 inactivate proteases with primer concentrations of 0.4 µM, 0.125U Taq polymerase (Fisher), and 0.2mM dNTP(PE/ABI.) Two cycle PCR was used, with an initial heat denaturation step at 94°C for 1' 40" a further denaturing step at 96°C for 20 seconds, then 30 cycles of 96°C for 16" and 68°C for 1' with a final extension of 4' at 74°C. Five µl of 6X loading dye was added and 12.5 µl each were run out on a 1.2% agarose gel. Results are
35 as follows: 12/18 positive colonies for spleen; 10/18 for bone marrow; 8/18 for lymph node;

9/18 for brain and 10/18 for adipose tissue. Two positive colonies each for the first 3 and 1 each for the latter two were grown up in 25 ml. liquid LB culture with 150 µg/ml ampicillin over night at 37°C with 250 rpm. Plasmid DNA was purified from the cultures with Qiagen's Tip-100 DNA purification kit per manufacturer's instructions. UV absorbance at 260nm was used to determine the plasmid yields which varied from between 131 and 169 µg of highly purified DNA.

Sequencing:

Sequencing reactions of double stranded plasmid DNA with Big Dye terminator cycle sequencing were performed in a 9700 thermocycler (Perkin Elmer/Applied Biosystems). Two vector primers (ICO383 and ICO384) and two PBGD specific internal primers (ICO380 and ICO381) were used for all 8 plasmids. In addition a fifth vector primer (ICO385) was used for the brain and adipose derived clones. Reaction conditions were per manufacturers protocol as follows: 500 ng plasmid DNA and 4 pmol oligonucleotide primer with 8 µl ready mix in 20 µl volumes with 30 cycles of 96°C for 12" and 60°C for 4'. Extension products were purified by isopropanol precipitation. To each reaction 20 µl of dH₂O and 60 µl isopropanol were added. These were mixed by inversion and allowed to sit at room temperature for 15 minutes then spun for 40' at 3250 rpm in a Beckman GS-6KR centrifuge with the GH3 rotor and Microplate + carriers. Reactions were inverted then spun at 1680 rpm for 1' to remove liquid from the pelleted DNA. DNA sequence analysis was performed at the University of Washington Biochemistry Department sequencing Laboratory with an Applied Biosystems 377 sequencer.

Analysis:

The inserts of all 8 clones were confirmed to be PBGD by complete double strand sequence analysis (see sequences 1 - 8). Each has some change(s) from the published sequences. Some changes are unique and some are shared with other clones (see Table 2 and Table 3). For differences found only in one clone, it is difficult to distinguish between PCR or cloning artifacts and actual allelic variations without additional sampling. However, when the same base difference is found in more than one sequence it is unlikely to be from cloning errors. From the alignment of all 11 PBGD sequences a set of common bases emerged, the consensus or wild type allele sequence. Five of the eight clones (1.1, 1.3, 2.1, 3.3, and 5.3.) have the wild type amino acid sequence. Within this set with wild type

amino acid sequence, there is only one difference at the nucleic acid level. At position 555, 4 of the 5 sequences have a dGTP while 1 along with the published erythropoietic and genomic PBGD have a dTTP. These appear to be two common alleles, which result in no amino acid difference. There are 2 base changes between clone number 1.1 and the published erythropoietic PBGD. An adenine to guanine change at base 513 (Leu 171) is a silent mutation, which is also present in 9 out of the 11 sequences, compared. The second difference is a cytosine to adenine substitution at base 995 (Thr 332.) This is not a silent change, with a threonine to asparagine non-conservative mutation. It appears however that the difference is an error in the published erythropoietic PBGD sequence since all 10 other sequences have an adenine at this position. In addition to these natural variations, there are three additional silent mutations introduced during the cloning at positions 1017, 1018 and 1020 to create a *Mun* I site for future manipulations. The PBGD gene was ligated into pBluescript SK plasmid generating the pSK-PBGD 3988 bp plasmid, which was sequenced.

15

Conclusion:

For any recombinant therapeutic protein it is important that the wild type allele be used to reduce the potential for immunogenicity. We feel confident through our survey of the literature and analysis of PBGD sequence from different individuals that clone number 1.1 represents the most prevalent "wild type" allele in the population with respect to amino acid sequence. Clone number 1.1 contains the consensus wild type amino acid sequence and differs from the published erythropoietic PBGD sequence by only one amino acid. Because this difference is found in all the other PBGD clones besides the erythropoietic PBGD sequence, it, rather than the published erythropoietic sequence, is deemed to be the prevalent wild type sequence. For this reason PBGD encoded by clone number 1.1 was chosen for production of recombinant human porphobilinogen deaminase (rhPBGD). In the following, the plasmid encoding the human wild type PBGD in clone number 1.1 will be termed "pPBGD1.1".

30

Equipment and supplies lists are shown in appendix 1 and 2, respectively.

Appendix 1 Equipment list

Item	Manufacturer	Serial Number
Pipetman P-1000	Gilson	N55287E

Pipetman P-200	Gilson	N52324E
Pipetman P-20	Gilson	N53465M
Pipetman P-10	Gilson	P626586
5415C centrifuge	Eppendorf	5415B68381
GS-6KR centrifuge	Beckman	NGD97J18
Avanti J-25 I centrifuge	Beckman	JJY97J14
DU 640B Spectrophotometer	Beckman	4323015
Genie II vortex	VWR	2-241186
GeneAmp PCR system 2400	Perkin Elmer (PE) / Applied Biosystems (ABI)	803N6021903
GeneAmp PCR system 2400	PE / ABI	803S7100104
GeneAmp PCR system 9700	PE / ABI	805S7121566
1545 incubator	VWR	0902597
heat block 1	VWR	0795
heat block 1	VWR	0511
Gene Pulser II Apparatus	BioRad	340BR2745
Pulse Controller Plus	BioRad	339BR1377
Power Pac 1000	BioRad	286BR00770
Sub Cell	BioRad	16S/8860
Wide-Mini Sub Cell	BioRad	02S/7951
Foto/Prep transilluminator	Fotodyne	PTG1-0997-2831
Elutrap Electro-separator	Schleicher + Schuell	Order No. 57880
Innova 4000 incubator	New Brunswick Scientific	890165366
Power Mac G3 computer	Macintosh	XA8061A3BBW
Trinitron Multiscan 200GS monitor	Sony	8057052
DNA analysis Software: Geneworks	Intelligenetics	Version 2.5.1

Appendix 2 Supplies List

5

Item	Supplier	Cat No.	Lot No.
Human Spleen Poly A+ RNA	Clontech	6542-1	7120266
Human Bone Marrow Poly A+	Clontech	6573-1	56714

RNA			
Human Lung Poly A+ RNA	Clontech	6524-1	7050104
Human Lymph Node Poly A+ RNA	Clontech	6594-1	6120292
Human Brain Poly A+ RNA	Clontech	6516-1	63101
Human Adipose Total RNA	Clontech	D6005-01	7907005
Superscript II reverse transcriptase	Gibco/BRL	18064-014	JM6418
100 mM dNTP set	Pharmacia	27-2035-01	6072035011
pBluescriptII SK- phagemid	Stratagene	212206	0270702
Advantage cDNA polymerase mix	Clontech	8417-1	8060354
GeneAmp dNTP	PE/ABI	N-808-0007	H0172.4,H0553
Xba-I endonuclease	New England Biolabs (NEB)	145S	30
Pvu-II endonuclease	NEB	151L	14
EcoR-I endonuclease	NEB	101L	25
Hind-III endonuclease	NEB	104S	49
Tris six-Pack "C"	Sigma	T-PAC-C	77H9049
0.5 M EDTA pH 8.0	Sigma	E-7889	16H8924
Chromaspin TE 400	Clontech	K1323-1	7090795
Chromaspin 400 DepC dH ₂ O	Clontech	K1333-1	7040086
Quiaquick gel extraction kit	Qiagen	28704	BY97017/0397/119
Microcon-30	Amicon	42410	L8JM4330B
Microcon-100	Amicon	42413	L8DM3296A

Micropure 0.22µm	Amicon	42544	CCB017
Seakem GTG agarose	FMC	50074	709397
100 bp DNA Ladder	NEB	323-1	3
123 bp DNA Ladder	Gibco/BRL	15613-029	JK9706
T4 DNA Ligase	NEB	202S	64
Ampicillin	Sigma	A-9518	76H0434
LB media	Gibco/BRL	12795-084	12E1072B
Bacto Agar	Difco	0140-07-4	106728JA
DH10B electromax	Gibco/BRL	18290-015	KHN430
SOC media	Gibco/BRL	15544-042	1010351
Taq polymerase	Fisher	FB-6000-15	H0436
TaqStart antibody	Clontech	5400-1	6070479
Qiafilter Midi DNA isolation kit	Qiagen	12243	PO No. 514
Isopropanol	Sigma	I-9516	47H3724
Big Dye terminator cycle sequencing kit	PE/ABI	4303152	9803008

Table 1 Oligonucleotide primers used for PCR amplification and sequencing of PBGD:

5 Ico375-pbgds (32 mer) coding region 5' end w/ EcoRI site sense

5' CGT GGA ATT CAT GAG AGT GAT TCG CGT GGG TA 3'

Ico376-pbgda (47 mer) coding region 3'end w/ HindIII site antisense

5' GGA GAA GCT TAT TAA TGG GCA TCG TTC AAT TGC CGT GCA ACA TCC AG 3'

10

Ico379-esnonc (20 mer) erythropoietic form non-coding sense

5' TCG CCT CCC TCT AGT CTC TG 3'

Ico380-sinter (21 mer) internal coding sense

15 5' CAG CAG GAG TTC AGT GCC ATC 3'

Ico381-ainter (21 mer) internal coding antisense

5' GAT GGC ACT GAA CTC CTG CTG 3'

Ico382-anonc (20 mer) non-coding antisense

5' CAG CAA CCC AGG CAT CTG TG 3'

Ico383-pSKT7 (22 mer) pBluescript T7 promoter

5' GTA ATA CGA CTC ACT ATA GGG C 3'

Ico384- pSKpjrev (22 mer) pBluescript reverse1

5' CTA AAG GGA ACA AAA GCT GGA G 3'

10 Ico385- pSKrev (21 mer) pBluescript reverse2

5' CAG CTA TGA CCA TGA TTA CGC 3'

Table 2 Variation of PBGD clones from published erythroid sequence:

PBGD clone	Differences from Erythroid mRNA			Genebank No.	Reference/Source
	silent	non-silent	total diffs		
Erythroid	0	0	0	X04217	Raich.N. et. al. 1986, Nucleic Acids Res. 14 (15), 5955-5968
Constitutive	1	2	3	X04808	Grandchamp.B. et. al. 1987, Eur. J. Biochem. 162 (1), 105-110
Genomic	1	2	3	M95623	Yoo,H.W. et. al. 1993, Genomics 15 (1), 21-29
1.1	1	1	2	-	Spleen (Clontech mRNA Lot No. 7120266)
1.3	2	1	3	-	Spleen (Clontech mRNA)
2.1	2	1	3	-	Bone Marrow (Clontech mRNA)
2.2	2	2	4	-	Bone Marrow (Clontech mRNA)
3.1	2	4	6	-	Lymph Node (Clontech mRNA)
3.3	3	1	4	-	Lymph Node (Clontech mRNA)
5.3	2	1	3	-	Total Brain (Clontech mRNA)
6.1	3	2	5	-	Adipose Tissue (Clontech mRNA)

15

Table 2:

Summary of the number of differences in amino acid sequence of our sequenced PBGD clones and clones from Genebank entries for the constitutive and genomic PBGD with

published Erythropoietic PBGD sequence. Shown in different columns are the total number of silent mutations with a DNA base change not causing a corresponding amino acid change, the number of non-silent mutations with a DNA change causing an amino acid difference and the sum of the two types of mutations. Not included in this table are the

5 three silent mutations introduced into the clones to create an internal *Mun* I endonuclease cleavage site. Note that clone number 1.1 which will be used for production of recombinant human porphobilinogen deaminase (rhPBGD) has only one of each type of difference with the least number of total differences.

Table 3 Summary of mutations found in PBGD clones:

aa	aa No.	bp No.	mutation	aa change	cons.	gen.	1.1	1.3	2.1	2.2	3.1	3.3	5.3	6.1	No. /10
Asp	19	56	A→G	Asp→Gly							X				1
Phe	108	322	T deletion	frame shift						X					1
Lys	140	419	A→G	Lys→Arg							X				1
Leu	160	478	C→A	Leu→Met		X									1
Ala	168	503	C→T	Ala→Val							X				1
Leu	171	513	A→G	silent		X	X	X	X	X	X	X	X	X	9
Val	185	555	T→G	silent	X			X	X	X		X	X	X	7
Glu	193	577	G→A	Glu→Lys	X							X			1
Gly	243	729	C→T	silent											1
Ala	280	840	T→C	silent										X	1
Ala	286	856	G→A	Ala→Thr										X	1
Lys	328	984	A→G	silent							X				1
Thr	332	995	C→A	Thr→Asn	X	X	X	X	X	X	X	X	X	X	10
Gln	339	1017	G→A	silent			X	X	X	X	X	X	X	X	8
Gln	339	1018	C→T	silent			X	X	X	X	X	X	X	X	8
Leu	340	1020	T→G	silent			X	X	X	X	X	X		X	7
Leu	340	1020	T deletion	frame shift									X		1

Table 3: Summary of the genetic differences of our sequenced PBGD clones and Genbank entries for the constitutive and genomic PBGD with published erythropoietic PBGD sequence from the allele sequence alignment. Listed in different columns are the amino acid, base number from the ATG start codon, the actual genetic difference with corresponding amino acid change if any and a listing of the clones with differences shown with an X. In the final column the total number of clones with the different mutations are shown. The three mutations at position 1017, 1018 and 1020 are introduced with ICO376 during PCR amplification to create a *Mun* I endonuclease cleavage site. Note that clone number 1.1 which will be used for production of rhpBGD only has genetic differences which also are represented by a number of other clones.

Expression plasmids

Construction of the basic expression plasmid pExp0

The basic expression plasmid pExp0 was constructed by excising the PBGD coding
 5 sequence (cDNA) from plasmid pPBGD1.1 (see Figure 1) with *EcoR* I and *Hind* III and
 inserting it into the vector pKK223-3 (Pharmacia, Catalogue # 27-4935) cut with the same
 enzymes, thus operatively linking it to the IPTG-inducible *tac* promoter (Amann E. et al.
 1983, Gene 25(2-3):167-178). Figure 1 shows the construction details. Plasmid pExp0 was
 constructed for a preliminary assessment of the expression levels and does not directly
 10 lead to the construction of the final expression plasmid.

Construction of the final expression plasmid

The final expression plasmid pExp1-M2-BB (Figure 9) was constructed in a multi-step
 process. The individual steps used and all the intermediate plasmids are outlined below.

15

Construction of plasmid pExp1

Plasmid pExp1 was first constructed with modifications to the 5' untranslated region and
 the initial part of the coding sequence both aimed at improving translation efficiencies (Gold
 L. and Stormo G.D. 1990, Methods Enzymol 185:89-93). The changes are indicated below,
 20 and include, insertion of a second ribosome binding site, an AT-rich sequence preceding
 the ATG and three silent base substitutions shown in boldface.

AATTCTAACA TAAGTTAAGG AGGAAAAAAA A ATG AGA GTT ATT CGT GTC GGT AC
 Met-Arg-Val-Ile-Arg-Val-Gly

25

A naturally occurring *Kpn* I site six amino acid residues into the coding sequence of the
 human cDNA for PBGD (pPBGD1.1) was exploited for this purpose. Oligonucleotides
 ICO386 and ICO387 were designed to provide upon annealing to each other a 5' *EcoR* I
 adhesive end and a 3' *Kpn* I sticky end and the elements described above including the
 30 codons for the first six amino acid residues as shown. Oligonucleotides ICO386 and
 ICO387 were annealed and ligated with the *Kpn* I-*Hind* III, PBGD fragment from pPBGD1.1
 into *EcoR* I-*Hind* III linearised pBluescript II SK- (Stratagene, Catalogue # 212206) to yield
 plasmid pPBGD1.1Tra. In the second step, the *EcoR* I-*Hind* III fragment from
 pPBGD1.1Tra was ligated into pKK223-3 cut with the same enzymes resulting in plasmid
 35 pExp1 (Figures 4 and 5).

Construction of plasmid pExp1-M2

The tetracycline resistance gene was next restored using the following strategy. Plasmid pExp1 was cut with *Sal* I and *Bam*H I and the 5349 base-pair fragment containing part of the tetracycline coding sequence and the bulk of the plasmid was isolated. Into this was ligated the *Sal* I-*Hind* III fragment from pBR322 (New England BioLabs, Catalogue # 303-3S) containing rest of the coding sequence and an adapter formed by annealing oligonucleotides ICO424 and ICO425 to each other. The adapter contains part of the tetracycline promoter and provides *Hind* III and *Bam*H I overhangs for ligation but destroys the *Hind* III and *Bam*H I restriction sites. The resulting plasmid was called pExp1-M2 (Figures 6 and 7).

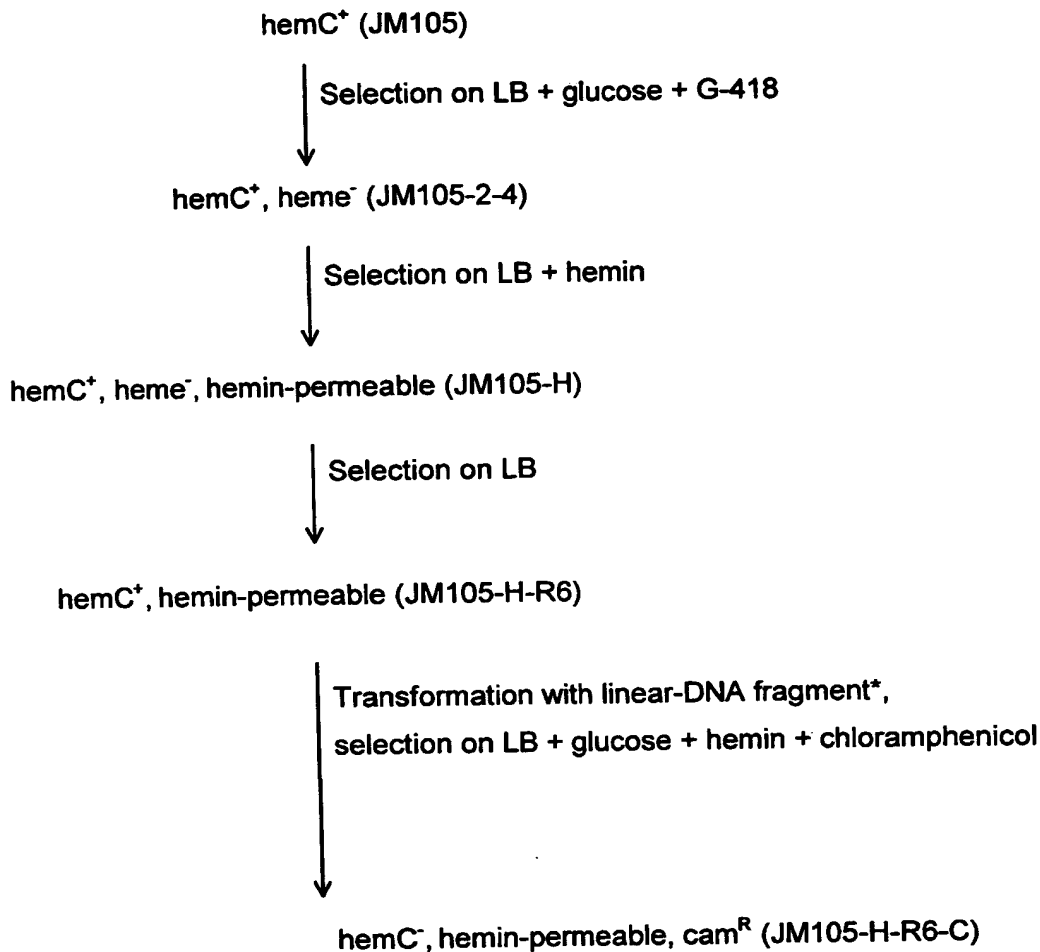
Construction of plasmid pExp1-M2-BB

In the final step the *rop* gene contained between *Bsa*A I and *Bsa*BI was deleted to increase copy number (Makrides S.C. 1996, Microbiol.Rev. 60(3):512-538). For this the plasmid pExp1-M2 was cycled through the *dam* minus strain, JM110 (F' [*tra*D36 *pro*A⁺ *pro*B⁺ *lac*I^r Δ (*lac*Z)M15] *dam* *dcm* *sup*E44 *hsd*R17 *thi* *leu* *thr* *rps*L *lac*Y *gal*K *gal*T *ara* *ton*A *tsx* Δ (*lac-pro*AB) *lambda*⁻) as restriction with *Bsa*B I is blocked by overlapping *dam* methylation. It was then cut with *Bsa*A I and *Bsa*B I to excise the *rop* gene and the 5446 base-pairs long linear fragment was circularised by blunt-end ligation to yield the production plasmid pExp1-M2-BB (Figures 8 and 9).

Construction of the *hemC*-deletion host and the final expression strain

The parent strain JM105 (F' [*tra*D36 *pro*A⁺ *pro*B⁺ *lac*I^r Δ(*lac*Z)M15] Δ(*pro-lac*) *hsd*R4 *sbc*B15 *rps*L *thi* *end*A1 *lambda*⁻), a derivative of *E. coli* K12 was obtained from Pharmacia, Catalogue # 27-1550-01. The *hemC* gene coding for the endogenous *E. coli* Porphobilinogen Deaminase was partially deleted. This was necessary to ensure that the purified product (rhPBGD) was free from contaminating *E. coli* PBGD as the *E. coli* and human enzymes are very similar in properties (Jordan P.M. 1994, Wiley, Chichester (Ciba Found Symp 180), p70-96) and may co-purify. The *hemC*-deletion host was derived from JM105 according to Scheme A. First a hemin-permeable variant was obtained by a three-step process. This was essential as a *hemC*-deletion mutant would require hemin for good growth and *E. coli* K12 strains are not freely permeable to hemin.

Scheme A. Scheme for obtaining *hemC*-deletion strain:



All mutant isolation was spontaneous. Approximately 5×10^{-8} - 5×10^{-9} cells were plated on
5 selective media. The media compositions are included as Appendix 1.

* For details on the linear DNA-fragment see Figure 11.

In the first step, a heme-minus mutant was isolated carrying a defect in any of the
10 biosynthetic steps leading to the formation of heme. Heme⁻ strains fall into the general
class of respiration deficient mutants that are defective in active transport and
consequently resistant to low levels of antibiotics of the aminoglycoside family such as
gentamicin (Lewis L.A. et al. 1991, Microbiol.Immunol. 35(4):289-301). Several
spontaneous mutants were isolated as a dwarf colonies on LB+glucose +G-418

- (gentamicin)-containing plates (Lewis L.A. et al. 1991, Microbiol. Immunol. 35(4):289-301). These were screened for their ability to respond weakly to hemin, indicating that they were heme⁻ (as opposed to other respiration deficient mutants which would not respond to hemin at all). One such heme-strain (JM105-2-4, see Scheme A) which could also revert back
- 5 spontaneously to robust growth on LB (as this is essential for the third step, see below) was selected. This strain was next plated on LB+hemin to obtain a better grower in the presence of hemin and was called JM105-H. It showed improved growth only in the presence of hemin, which meant that it still was heme⁻ but had become hemin-permeable. To restore the functionality of the heme biosynthetic pathway in JM105-H, spontaneous
- 10 revertants were isolated on LB plates and only those retained which resembled the starting strain JM105 in growth, both untransformed and after transformation with the expression plasmid. One such strain used in this study was called JM105-H-R6 and should have retained the heme-permeable trait of its parent strain.
- 15 Strain JM105-H-R6 was transformed with the *EcoR* I-*Hind* III fragment (see Scheme A); to obtain the *hemC*-deletion host called JM105-H-R6-C by homologous gene replacement. This strain has the genotype, F'[*traD36 proA⁺ proB⁺ lacI^q Δ(lacZ)M15*] *Δ(pro-lac) hsdR4 sbcB15 rpsL thi endA1 lambda⁻ hemC::CAT* hemin-permeable. It was transformed with the expression plasmid pExp1-M2-BB to yield the final production strain PBGD-2 (PBGD-2
- 20 was deposited under the Budapest Treaty on 9 July 1999 with DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany) under the accession No. DSM 12915).

- In order to obtain the *EcoR* I-*Hind* III fragment, a multiple PCR strategy was used.
- 25 Oligonucleotide pairs ICO437, ICO438 and ICO505, ICO440 were used to amplify separately, portions of *E. coli* JM105 genomic DNA segments flanking the *hemC* gene (see Figure 10). These amplified gene products were digested with pairs of enzymes *EcoR* I, *Xho* I and *Xho* I, *Hind* III respectively, and in essence, assembled together between the *EcoR* I and *Hind* III sites of pUC19 to give plasmid phemCd. Next the fragment containing
- 30 the chloramphenicol-resistance gene was PCR amplified from plasmid pBC SK⁺ (Stratagene, Catalogue # 212215) using oligonucleotides ICO510 and ICO511. This product was cut with *Xho* I and inserted into plasmid phemCd at the *Xho* I site. In essence, the plasmid having the Cam gene in the orientation shown was called phemCdCm and formed the source of the *EcoR* I-*Hind* III linear DNA-fragment depicted in Figure 11.

In Figure 11 the structure of the linear DNA-fragment used for the transformation is shown. The genetic organization of the *E. coli* polypeptides depicted by gray arrows (*cyaA*, *hemC*-5', *hemC*-3', *hemX* and *hemD*) is derived from the GenBank report Accession number AE000456. The black arrow represents the 767 base-pairs long PCR fragment carrying the 5 cholamphenicol-resistance gene (*Cam*), encoding chloamphenicol acetyltransferase (*CAT*), replacing 806 base-pairs of the *hemC* coding sequence. *HemC*-5' and *hemC*-3' correspond to 149 and 16 base-pairs respectively, of the coding sequence of the disrupted *hemC* gene. *EcoR* I, *Xho* I and *Hind* III are engineered restriction sites. The sequence of this 3225 base-pairs long fragment is shown in Seq. ID NO 2. The two *Xho* I sites are at positions 10 1072 and 1839 in the sequence, respectively.

Table 4 Oligonucleotide primers used in the construction of the production strain PBGD-2

	ICO386	(54 mer)	Construction of plasmid pExp1
15	5' AAT TCT AAC ATA AGT TAA GGA GGA AAA AAA AAT GAG AGT TAT TCG TGT CGG TAC 3'		
	ICO387	(46 mer)	Construction of plasmid pExp1
	5' CGA CAC GAA TAA CTC TCA TTT TTT TTT CCT CCT TAA CTT ATG TTA G 3'		
20	ICO424	(32 mer)	Construction of plasmid pExp1-M2
	5' GAT CAC TCA TGT TTG ACA GCT TAT CAT CGA TT 3'		
	ICO425	(31 mer)	Construction of plasmid pExp1-M2
25	5' AGC TAA TCG ATG ATA AGC GTC AAA CAT GAG T 3'		
	ICO437	(32 mer)	Amplification of product P1
	5' AGT CAG AAT TCA GAC GCA CGG CGG TAC GAT AA 3'		
30	ICO438	(32 mer)	Amplification of product P1
	5' ATT CAC TCG AGG TCA CCA TCG GTA CCA GTT CA 3'		
	ICO440	(32 mer)	Amplification of product P2
	5' AGA TCA AGC TTC GGC CAG ACG CAG GTT ATC TA 3'		
35			

ICO505 (34 mer) Amplification of product P2
5' ATA CAC TCG AGA CCG GCA TGA GTA TCC TTG TCA C 3'

ICO510 (30 mer) Amplification of Cam gene
5' ACT GAC CTC GAG CGG CAC GTA AGA GGT TCC 3'

ICO511 (29 mer) Amplification of Cam gene
5' ACT GAA CTC GAG AAT TAC GCC CCG CCC TG 3'

10 Accordingly, the cDNA used for expressing rhPBGD was derived from plasmid pPBGD1.1. The starting host strain was derived from JM105 and is called JM105-H-R6-C. The genotype and the details on its construction are described above. A large part of the coding region of the *hemC* gene was replaced by the Cam gene, encoding chloramphenicol acetyltransferase. This gene replacement was confirmed by PCR amplification of the
15 segment of the *E. coli* genome followed by restriction analysis of the amplified product. As a result of the gene replacement, the strain is resistant to chloramphenicol and grows extremely poorly on LB medium. Growth improves when LB medium is supplemented with hemin.

20 Expression construct

The expression plasmid in the final production strain is pExp1-M2-BB. Its construction is described above. A detailed map of the plasmid showing the open reading frames and functionally relevant regions is shown in Figure 9. The complete DNA sequence is included in Seq. ID NO 1. All synthetic adapters and linkers used during the construction have been
25 sequenced along with all junctions created during ligation which directly impinge upon the expression of the cloned gene.

Production strain

The final production strain is called PBGD-2. It was obtained by introducing the expression
30 plasmid pExp1-M2-BB into the host strain JM105-H-R6-C, essentially, by rendering the cells competent with 100mM CaCl₂ (Morrison D.A. 1979, Methods.Enzymol. 68:326-331) and selecting for transformants on LB + ampicillin media at 30°C. The plasmid is a derivative of pBR322 without the *rop* gene and should be present extrachromosomally in moderate copy number at 30°C with a slightly higher copy number at elevated
35 temperatures 37°C and greater (Makrides S.C. 1996, Microbiol.Rev. 60(3):512-538). It has both the ampicillin and tetracycline resistance genes as selectable markers. It also

expresses rhPBGD which can complement the *hemC* defect of the host strain. As a result, the production strain should grow normally on LB/M9 media, be resistant to the antibiotics ampicillin and tetracycline and also be resistant to the antibiotic chloramphenicol (because of the presence of the Cam gene in the genome). It was confirmed to have all these

5 characteristics.

Expression

The expression of rhPBGD is driven by the *tac* promoter which is regulated by the copy of the *lacI^d* gene present in the host. Due to the modifications made to the system as

10 described in the study plan, the uninduced level of expression is 1.8 units/mg (see Appendix 3 for assay details), which amounts to approximately 10% of the total soluble protein. The culture is grown throughout at 30°C and no induction step is used to increase expression.

Evaluation and conclusions

15 The expression system developed for the production of rhPBGD in *E. coli* is a stable system, producing good amounts of rhPBGD in a constitutive manner when the cells are grown at 30°C. The host strain employed is partially deleted for the gene producing the endogenous *E. coli* porphobilinogen deaminase. After transformation with the expression plasmid, the resulting production strain PBGD-2 grows as well as the strain PBGD-1 (which

20 is JM105 carrying the same expression plasmid) and makes the same amount of rhPBGD.

Alternative expression construct:

Expression plasmid pExp1-M2-Puc-BB and expression of rhPBGD in *E. coli*

25

The plasmid pExp1-M2 was digested with Pvu I and Afl III and the larger of the two fragments corresponding to a size of 4745 base-pairs was isolated. This was ligated to the 1257 base-pairs long Pvu I-AflIII fragment derived from pUC19 containing the origin of replication and part of the ampicillin resistance gene to obtain plasmid pExp1-M2-Puc. This

30 was passaged through JM110 and cut with BsaA1 and BsaB1 to excise the *rom* gene contained between the two sites and blunt-ended together to yield the final expression plasmid pExp1-M2-Puc-BB. The pExp1-M2-Puc-BB plasmid has been fully sequenced and differs from pExp1-M2-BB only in that C in position 2769 is T in pExp1-M2-Puc-BB.

35 Expression of rhPBGD in *E. coli*

The *E. coli* K12 host strain JM105 genotype endA thi rpsL sbcB15 hsdR4 $\Delta(\text{lac-proAB})$ [F'traD36 proAB lacI^q $\Delta(\text{lacZ})$ M15] containing the expression plasmid pExp1-M2-Puc-BB was grown in LB broth containing 100 $\mu\text{g/ml}$ ampicillin at to mid-log phase at 30°C from a 1 to 40 dilution of an overnight inoculum. The culture was then split into three and growth was continued for another 4 hours at 30°C, 37°C and 42°C respectively. Cells were spun down from 1 ml samples and frozen at -20°C. The thawed cell pellets were resuspended in 200-300 μl of B-PER reagent PIERCE Cat. # 78243, incubated at room temperature for 10 minutes, spun at 16,000 for 10 minutes and PBGD activity was determined in the supernatants. Total protein was estimated by the Bradford method using the BioRad reagent Cat # 500-0006 and bovine serum albumin as standard. The specific activities in the crude lysates obtained at the three growth temperatures are tabulated below. The results clearly show an increase of PBGD units/mg with increasing temperature in the range from 30°C to 40°C.

15 Temperature	PBGD Units/mg
30°C	363
37°C	573
42°C	1080

20 Other Production Systems For rhPBGD

For yeast production, the PBGD coding sequence can be inserted into a plasmid vector, for example YEP type, containing 2 u circular DNA (Ori) origin for high expression in yeast. YEP plasmids contain TRP 1 and URA 3 as markers for selective maintenance in trp1-, ura

25 3-yeast strains.

Alternatively, the PBGD gene can be inserted in bovine papilloma virus vectors BPV for high expression in a murine cell line C-127 (Stephens P.E. et. al. Biochem J. 248, 1-11, 1987) or vectors compatible with expression in CHO cells or COS cells.

30

An expression of PBGD can be made intracellularly.

A secretory signal in *Saccharomyces*, for example alpha-mating factor presequence, can be added in front of the rhPBGD structural gene for efficient secretion in yeast.

Similarly, a sequence encoding a mammalian signal peptide can be added for secretion of rhPBGD into the culture medium upon expression in for example CHO cells or COS cells.

A bacterial promoter for example the tryptophane (trp) promoter or the lac promoter or
5 alternatively an alkaline phosphatase promoter, should be inserted before the PBGD coding region for efficient transcription in prokaryotes for example E. coli.

A yeast promoter for example 3-phosphoglycerate kinase (PGK) or chelatin or alpha-mating factor should be inserted before the PBGD coding region for efficient transcription in
10 yeast for example *Saccharomyces cerevesiae* or *Saccharomyces pombe*.

A mammalian promoter for example Metallothionin-1 (MT-1) or Aspartate transcarbamylase or Dihydrofolate reductase (DHFR) should be inserted before the PBGD coding region for efficient transcription in mammalian cell lines for example CHO cells or
15 COS cells.

The yeast plasmid (Y-G&F-PBGD) containing a yeast promoter, signal and/or ATG codon (methionine) in front of the PBGD coding region and a yeast vector containing selectable markers such URA 3 or TRP 1 will be transformed into the yeast host cell such as
20 *Saccharomyces cerevesiae* or *Saccharomyces pombe* for production of rhPBGD.

The mammalian plasmid (M-G&F-PBGD) containing a mammalian promoter for example Metallothionine-1 or Dihydrofolate reductase and a mammalian signal sequence or an ATG codon in front of the PBGD coding region and vector pAT or pSV2 respectively. Plasmid
25 (M-G&F PBGD) may be transfected into a mammalian cell line for example CHO cells, for production of rhPBGD.

The E. coli cell containing plasmid (pExp1 or pExp1-M2 Puc-BB), may be fermented to stationary phase between 24-48 hours, in a medium containing casein hydrolysate, or
30 yeast extract, glucose, vitamins and salts. pH oxygen may be monitored by electrodes during fermentation. Temperature will be kept at 37 +/- 2 C during the fermentation.

The yeast cell containing the plasmid (Y-G&F-PBGD), may be fermented to late log phase between 20-40 hours in a medium containing yeast extract, glucose, salts and vitamins. pH

and temperature will be monitored by specific electrodes during fermentation. Temperature will be kept at 30+/- 2 C during fermentation.

The mammalian cell line containing the plasmid (M-G&F-PBGD) may be fermented in a medium containing, foetal calf serum (or serum free), vitamins, glucose, antibiotics, growth factors. pH and temperature will be monitored continuously during fermentation by specific electrodes.

Fermentation and Purification

rhPBGD may be recovered from E. coli after fermentation by an extraction procedure involving for example ribipress, sonication, osmotic shock or total solubilization by detergent for example Tween 80, Triton X-100 or Brij. rhPBGD will be recovered from fermentation medium after production in yeast or from a total cellular extract using detergents such as Triton X-100, Tween 80 or Brij. rhPBGD will be recovered from mammalian culture medium or from a total cellular extract by ion-exchange chromatography or affinity chromatography.

rhPBGD may be purified from E. coli extract or from yeast medium or total cellular extract or from mammalian culture medium or total mammalian cellular extract by binding to an ion-exchange column for example DEAE-Sepharose or MonoQ-Sepharose and eluted with for example NaCl and Sodium phosphate buffer pH 7-8 or the corresponding potassium salts.

Alternatively, rhPBGD may be recovered from extracts by binding to an affinity chromatography column for example an anti-PBGD affinity column. rhPBGD will be eluted by lowering the pH to 4-2, or a thiol specific affinity column. rhPBGD has been "tagged" with thiols residues when a thiol affinity column step is used. Thiols will be removed by a specific enzymatic cleavage step to generate authentic rhPBGD.

The ion-exchange or affinity purified rhPBGD will be further purified by hydrophobic interaction chromatography on for example, TSK Phenyl 5 PW column or Octyl-Sepharose or Phenyl-Sepharose columns.

Binding of rhPBGD may be done at high ionic strength for example in 10-50 mM Tris-HCl pH 7-8, 1M NaCl or 10-15 mM Sodium phosphate pH 7-8, 0.5 M MgSO₄ and eluted by

lowering the ionic strength for example with 10-50 mM Tris-HCl pH 7-8 or 10-50 mM Sodium phosphate pH 7-8.

Three hydrophobic interaction steps will be applied consecutively.

5

rhPBGD is further purified with preparative RP-HPLC for example C12 or C18 matrixes. The rhPBGD is be eluted from the column by a gradient of 10-50 mM Sodium phosphate and 1-10% acetonitrile buffer.

10 Formulation of rhPBGD is done by passing the enzyme over a G-100 Sephadex column and eluting it in an isotonic solution for example 0.9%NaCl and 10-50mM Sodium phosphate pH7.0 +/- 0.5 or Sodium phosphate, glycin, mannitol or the corresponding potassium salts.

15 For the preparation of a medicament, the formulation solution of rhPBGD may be sterile filtered and filled aseptically in glass vials and lyophilised.

Alternatively, the sterile filtered rhPBGD solution is formulated in for example, lipid vesicles constituting phosphatidylcholine or phosphatidylethanolamine or combinations of these or

20 incorporated into erythrocyte ghosts.

Reconstitution of lyophilised rhPBGD may be done in water for injection.

Alternatively, rhPBGD is formulated in a sustained release formulation involving a

25 biodegradable microspheres, for example in polylactic acid, polyglycolic acid or mixtures of these.

Alternatively, rhPBGD is lyophilized in a two-compartment cartridge, where rhPBGD will be in the front compartment and water for reconstitution in the rear compartment. This two

30 compartment cartridge may be combined with an injection device to administer either rhPBGD by a needle or needle less (by high pressure) device.

Alternatively, rhPBGD may be formulated in a physiological buffer containing an enhancer for nasal administration.

35

Alternatively, rhPBGD is formulated in an oral formulation containing for example, lipid vesicles (phosphatidylcholine, phosphatidylethanolamine, sphingomyeline) or dextrane microspheres.

- 5 Although recombinant production of PBGD is preferred for the treatment of AIP, it can alternatively be produced from human red blood cells.

A general production and manufacturing of recombinant PBGD may be done by the following steps.

10

Recombinant PBGD production process; an outline

A: Fermentation

1. Master cell bank
- 15 2. Working cell bank
3. Production of seed culture
4. Fermentation in large fermenter (250 L >)

B. Purification

- 20 1. Cell concentration by filtration/centrifugation
2. Cell disruption
3. Ultrafiltration
4. Chromatography ion exchange, DEAE-Sepharose, MonoQ-Sepharose
5. Hydrophobic interaction chromatography (Octyl/phenyl-Sepharose, TSK Phenyl, 5PW,
- 25 Phenyl -Sepharose
6. Chromatography ion exchange (MonoQ)
7. Formulation by Gel filtration Sephadex G-100

C. Manufacturing

- 30 1. Sterile filtration
2. Aseptic filling
3. Lyophilization

35

Treatment of other porphyrias

In analogy with the new treatment of AIP patients with (recombinant) PBGD, hepatic Porphyrias such as ALA deficiency Porphyria (ADP), Porphyria cutanea tarda (PCT),

- 5 Hereditary Coproporphyria (HCP) and Variegata Porphyria (VP) can benefit from substitution therapy by rALA dehydratase, rUroporphyrinogen decarboxylase, rCoproporphyrinogen oxidase and rProtoporphyrinogen oxidase, respectively.

- 10 Patients having Erythropoietic Porphyrias such as Congenital erythropoietic Porphyria (CEP) or Erythropoietic protoporphyria (EPP) will benefit from substitution therapy with rUroporphyrinogen III syntetase and rFerrochelataase, respectively.

Hepatoerythropoietic Porphyrias e.g. Hepatoerythropoietic Porphyrias(HEP) can be treated with rUroporphyrinogen decarboxylase.

15

All porphyrias can be treated by the administration of the enzymatic activity lacking or being reduced (normally 50%) in any of the eight steps in the heme biosynthetic pathway as described above.

- 20 The substitution of the enzymatic activity can be achieved by adding the corresponding recombinant enzyme or other molecules that will provide the missing enzymatic activity.

Gene therapy as an alternative treatment for patients wiht acute intermittent porphyria (AIP)

25

The human enzyme Porphobilinogen deaminase PBGD is coded for by a single gene located on chromosome 11q 24.

- 30 Mutations in this gene causes the disease Acute Intermittent Porphyria (AIP). The disease has been shown to be inhereted in an autosomal dominat way.

Today over 100 mutations in the PBGD gene has been identified (Grandchamp B. J. Ganstroenterology and Hepathology, 11, 1046-1052, 1996, Table A) and the number is expected to increase when modern diagnostic systems based on screening programs will
35 be applied more routinely in hospitals. A number of these mutations are shown in Table A.

Table A Reported mutations in the PBGD gene

	Position	Mutation	Consequences	Reference
Exon 1	3	ATG→ATA	Translation impairment	18
	33	GCG→GCT	DS	17
<i>Intron 1</i>	33+1	gtg→atg	DS	16
Exon 3	76	CGC→TGC	R26C	25
	77	CGC→CAC	R26H	26
Exon 4	91	GCT→CACT	A31T	24
	97	Del A	Frameshift	25
	100	CAG→AAG	Q34K	27
	100	CAG→TAG	Q34X	25
	125	TTG→TAG	L42X	19
Exon 5	163	GCT→TCT	A55S	24
	174	Del C	Frameshift	24
	182	Ins G	Frameshift	24
<i>Intron 5</i>	210+1	gta→ata	DS (Del exon 5)	24
Exon 6	218-219	Del AG	Frameshift	24
Exon 7	277	GTT→TTT	V93F	24
	293	AAG→AGG	K98R	25
	331	GGA→AGA	G111R	28
<i>Intron 7</i>	345-1	cag→caa	AS (Del exon 8)	29
Exon 8	346	CGG→TGG	R116W	20
	347	CGG→CAG	R116Q	30
Exon 9	445	CGA→TGA	R149X	25
	446	CGA→CAA	R149Q	31
	446	CGA→CTA	R149L	24
	463	CAG→TAG	Q155X	32
	470	Ins A	Frameshift	29

Table A cont.

	Position	Mutation	Consequences	References
<i>Intron 9</i>	499-1	cag→caa	AS (Del exon 10)	21
Exon 10	499	CGG→TGG	R167W	33
	500	CGG→CAG	R167Q	27,34
	518	CGG→CAG	R173Q	34
	530	CTG→CGG	L177R	27
	593	TGG→TAG	W198X	19
	604	Del G	Frameshift	35
	610	CAG→TAG	Q204X	30
	612	CAG→CAT	DS (Del 9 bp exon 10)	31
Exon 11	625	GAG→AAG	E209K	28
<i>Intron 11</i>	652-3	cag→gag	AS (Del exon 12)	33
Exon 12	667	GAA→AAA	E223K	24
	673	CGA→GGA	R225G	25
	673	CGA→TGA	R225X	25
	713	CTG→CGG	L238R	25
	715-716	Del CA	Frameshift	19
	730-731	Del CT	Frameshift	36
	734	CTT→CGT	L245R	31
	739	TGC→CGC	C247R	36
	740	TGC→TTC	C247F	18
	742	Ins 8 bp	Frameshift	24
	748	GAA→AAA	E250K	24
	754	GCC→ACC	A252T	36
	755	GCC→GTC	A252V	36
	766	CAC→AAC	H256N	27
	771	CTG→CTA	DS (Del exon 12)	39
	771	CTG→CTC	DS (Del exon 12)	37
<i>Intron 12</i>	771+1	gta→ata	DS (Del exon 12)	19

Table A cont.

	Position	Mutation	Consequences	References
Exon 13	806	ACA→ATA	T269I	30
	820	GGG→AGG	G274R	30
Exon 14	838	GGA→AGA	G280R	25
	848	TGG→TAG	W283X	30
	886	CAG→TAG	Q296X	25
	900	Del T	Frameshift	31
Intron 14	912+1	gta→ata	DS (Del exon 14)	28
Exon 15	1062	Ins C	Frameshift	38
	1073	Del A	Frameshift	25

In one further aspect, the present invention relates to a therapeutic method for AIP patients based on gene therapy, preferably in combination with administration of a catalyst according to the present invention. The gene therapy treatment may involve the following steps:

1. Identification mutations in the PBGD gene causing AIP in humans
2. Selection of human PBGD cDNA sequence for gene therapy
- 10 3. Construction of PBGD gene therapy vectors.
4. Production of PBGD gene transfer vector
5. Delivery system of PBGD gene transfer vector

1. Identification of mutations in the PBGD gene causing AIP in humans

15

Patients having a point mutation in Exon 10 at position 593 TGG>TAG have a change in the amino acid sequence of the PBGD enzyme from W198X (stop codon). This mutation is carried by approximately 50 % of all AIP patients in Sweden (Lee JS. et al. Proc. Natl. Acad. Sci. USA, 88, 10912-10915, and 1991). AIP patients with other mutations than

20 W198X, which might also benefit from gene therapy, are given in Table A.

2. Selection of human PBGD cDNA sequence for gene therapy

There are two isoenzyme forms of human PBGD e.g. erythropoietic and the non-erythropoietic form, which are formed by an alternative splicing mechanism.

25

The non-erythropoietic form has a 17 amino acid extension on the N-terminal end of the erythropoietic PBGD form.

Non-erythropoietic PBGD form (nPBGD):

- 5 Met-Ser-Gly-Asn-Gly-Asn-Ala-Ala-Ala-Thr-Ala-Glu-Glu-Asn-Ser-Pro-Lys-Met-Arg-Val....
ATG-TCT-GGT-AAC-GGC-ATT-GCG-GCT-GCA-ACG-GCG-GAA-GAA-AAC-AGC-CCA-AAG-ATG-
AGA-GTG..

Erythropoietic PBGD form (ePBGD):

10

Met-Arg-Val-
ATG-AGA-GTG...

- The nucleotide and amino acid sequence for human PBGD that will be used for gene
15 therapy differs from that published by Raich N. et al. Nucl. Acid. Res. 14, 5955-5968, 1986
in that the amino acid residue in position 332 is an Asn residue rather than Thr. In order to
make the "wild type enzyme" and avoiding formation of antibodies the PBGD sequence has
to contain an Asn residue in position 332. The cDNA sequence that will be used for the
erythropoietic PBGD form is shown above.

20

Patient with a defect erythropoietic PBGD enzyme will be transfected with the
erythropoietic PBGD cDNA sequence and patients with a defect in the non-erythropoietic
form will be transfected with the non-erythropoietic cDNA sequence.

25 3. Construction of PBGD gene therapy vectors

Adenoviral vector system

- The vector is based on adenovirus type 5 (Ad5), containing three essential genetic loci
E.g. E1, E2, E4, encoding important regulatory proteins and one locus E3 which is non-
30 essential for virus growth. Deletion of E1A and E1B region renders the virus replication
deficient in vivo. Efficient complementation of the E1 function (recombinant viral stocks)
can be obtained in an E1 expressing cell line such as human 293-cell line.

The human PBGD cDNA will be inserted in an adenovirus vector system.

35

The PBGD transgenes will be driven by the endogenous PBGD promoter or a cytomegavirus promoter (CMV).

Retroviral vectors

- 5 Retroviral vectors are well suited for gene delivery for several reasons:
1. simplicity
 2. capacity to integrate up to 8kbp DNA inserts
 3. their safety, non pathogenic to humans
 4. easy to improve and manipulate
 - 10 5. defined integration sites of genes
 6. long term regulated expression

One major disadvantage with the retroviral vectors though, is that they can only transduce dividing cells.

15

Most common retroviridae considered for gene therapy, are the lentiviridae and the mammalian C-type viridae. Other type retroviruses have also been considered. One such example, is a Moloney-murine leukemia retrovirus (Mo-MLV), which has been successfully used to transduce mouse and human fibroblasts with the uroporphyrinogen III synthetase

20 (UROIIIIS). (Moreau-Gaudry et al. Human Gene Therapy 6, 13-20, 1995).

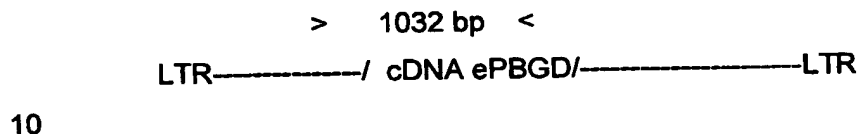
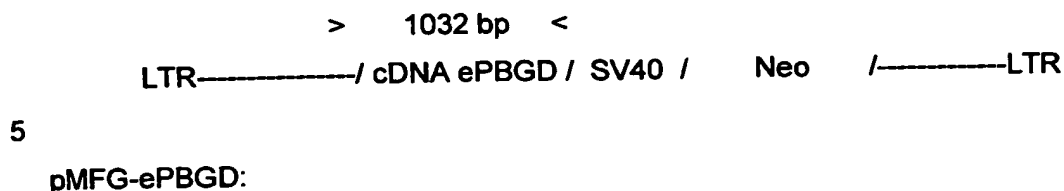
The expression of the UROIIIIS gene was driven by long terminal repeat (LTR). The UROIIIIS cDNA was also successfully transduced by the retrovirus vectors into human peripheral blood progenitor cells.

25

The erythropoietic PBGD cDNA sequence can be inserted in a retrovirus vector LXSN (Miller et al BioTechniques 7, 980-990, 1989) and pMFG (Dranoff et al. Proc. Natl. Acad.Sci. USA. 90,3539-3543, 1993). This will lead to the following constructs e.g. LePSN and pMFG-ePBGD, respectively.

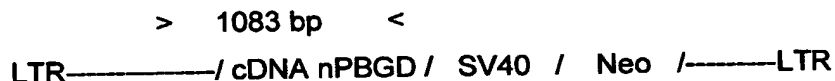
30

LePSN:



For transduction of non-erythropoietic tissues the non-erythropoietic cDNA (See sequence 12) will be inserted in the LSXN vector and the pMFG vector resulting in the LSnPN and pMFG-nPBGD vectors, respectively.

15 LnPSN:



20 pMFG-nPBGD:



- 25 The LePSN and LnPSN vectors can be converted to the corresponding virus by transfer into an appropriate host cell line e.g. Ψ CRE as described by (Danos et al. Proc. Natl. Acad. Sci. USA. 85, 6460-6464, 1988). Filtered supernatants from ectopic virus producing cells were added to amphotropic cells Ψ CRIP, in the presence of Polybrene. Clones can be isolated and tested for virus. Clones that show titers over 1.000.000 cfu/ml will be saved
- 30 (resistant to G418). The LnPSN vector will be cotransfected with the pMCI-Neo plasmid (Pharmacia, Sweden) into the packaging cell line Ψ CRIP. Clones that shows integration of provirus and high expression levels of message will be selected.

Filtrate from supernatants from virus producing cells (erythropoietic PBGD form) can be

35 mixed with Polybrene and incubated with peripheral blood progenitor cells (bone marrow

transplant) from an AIP patient for several hours. The transduced progenitor cells can then be transplanted back into an AIP patient.

The success of the treatment will be measured as the increase in the PBGD activity in erythrocytes and reduced excretion of ALA and PBG in the urine. Clinically a success of the treatment can be evaluated as a reduction of frequency of spontaneous acute attacks or drug-induced attacks. This will be a more convenient way of administering the recombinant PBGD enzyme than regular injections. The efficacy of the therapy can be evaluated by measuring the PBGD activity in blood and reduced excretion of PBG and ALA in the urine. Clinically, a successful treatment should result in less number of acute attacks or preferably no more attacks.

Associated Adenovirus system (AAV)

AAV is a non-pathogenic human virus (Parvovirus) carried by more than 80% of all people. The advantage with AAV as compared to retroviral systems is that AAV can transduce both dividing and non-dividing cells. The virus genome, which is small, contains two Inverted Terminal Repeats (ITR) and a REP and CAP functions. The REP and CAP functions can be deleted and exogenous cDNA inserted. Construction of an AAV vector containing the erythropoietic PBGD cDNA can be made. This AAV/PBGD vector will be suitable to transduce AIP patient's bone muscle cells, as a "muscle factory" for PBGD enzyme production. The PBGD cDNA will be engineered in such a way that a signal sequence for secretion will be added on the 5'-end of the cDNA. This will allow the erythropoietic PBGD enzyme to become secreted from the muscle cells into the blood stream. By this system patients will receive a constant delivery of active PBGD enzyme into the bloodstream, which will metabolize PBG thereby avoiding acute attacks.

- Non-erythropoietic

Alternatively, liver cells can be transduced with AAV containing the non-erythropoietic PBGD cDNA. The construct will be engineered in such a way that the translated PBGD enzyme will remain intracellular e.g. contain a Met residue at the N-terminal end of the PBGD enzyme without a signal sequence for secretion in mammalian cells. The PBGD transgene will be transcribed and translated into new PBGD enzymes that will remain intracellularly. Levels of new PBGD enzymes made in the liver will be normalized the PBGD activity to 100%. AIP patients have usually reduced PBGD activity (50-80%) in the liver depending on the mutation and individual variations.

This treatment would alleviate the clinical symptom e.g. acute attacks with abdominal pain and reduce excretion of PBG and ALA in the urine. The AAV containing the non-erythropoietic PBGD form can also be used to correct the genetic defect in other cell types such as neuronal tissue, pancreas spleen e.g. non-erythropoietic tissue, by a similar mechanism.

- Erythropoietic

The erythropoietic PBGD cDNA can be inserted in an AAV vector and used to transduce erythropoietic cells and stem cells in AIP patients, having a mutation affecting the erythropoietic form of PBGD.

4. Production of PBGD gene transfer vector

Adenovirus have approximately 36 kbp double stranded DNA, containing three essential early gene loci (E1, E2, and E4) encoding important regulatory proteins. Loci E3 codes for a gene product that block immune response to virus infected cells *in vivo*. The PBGD gene transfer adenovirus vector can be produced by deleting the E1 and E3 loci. The PBGD gene cassette is inserted in that position instead. The virus will be replication defective when the E1 locus has been deleted. Efficient E1 complementation and thus high yield of recombinant virus vector (PBGD) can be obtained in an E1 expressing cell line, such as the human 293 cell line. (Graham, F. et al. 1977, Characteristics of a human cell line transformed by DNA from human adenovirus 5. J. Gen. Virol. 36, 59-72).

5. Delivery systems of PBGD gene transfer vectors.

Delivery of viral vectors are based on injection into the patient of a virus particle that will transduce human cells *in vivo*.

Correction of point mutations causing AIP by Chimeraplasty Gene Repair

The basic technique involves the synthesis of chimeric (RNA-DNA) oligonucleotides. The oligonucleotide will repair point mutations on the chromosome by binding to the site of mutation and create a mismatch. The endogenous "mismatch repair system" which is present in all living cells, will correct the mutation.

The Chimeric oligonucleotides has the following general properties:

- a. 68 mer (65-70 is acceptable size)
- b. 25 base DNA stretches at the 5'-end homologous to the normal sequence of the gene
- 5 c. the 25 base DNA is designed in such a way the 12 bp on each side of the mutation is complementary to "wild-type DNA" where the mutation to be altered is located at position 13
- d. the 25 mere contains 4 T bases at the one end to loop back the oligo to the other DNA strand with a 25 base sequence homologous to the other strand of the chromosomal
- 10 DNA.
- e. the second strand is chimeric in that it contains 10 homologous bases of 2'O methyl RNA followed by 5 bases of DNA (containing a central mismatch e.g. correction of the human point mutation by mismatch repair) followed by another stretch of 10 bases of homologous 2'O methyl RNA. This stretch of DNA/RNA is followed with 5 bases of GC
- 15 clamp and 4 T bases to form the second loop and finally a 5 base CG clamps complementary to the other one.

EXAMPLE A

20 Correction of the PBGD mutation at position 593 TGG>TAG resulting in W198X

Normal Chromosomal Sequence:

5'-AG CGC ATG GGC TGG CAC AAC CGG GT-3'

Gln Arg Met Gly Trp His Asn Arg Val

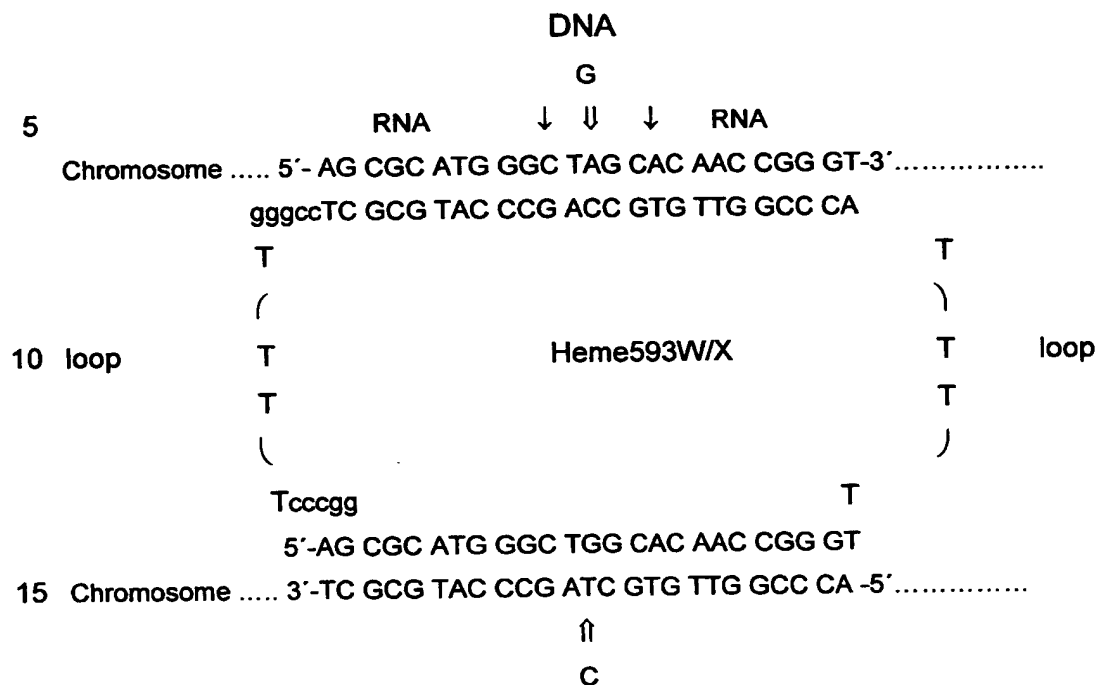
25

AIP Chromosomal Sequence:

5'- AG CGC ATG GGC TAG CAC AAC CGG GT-3'

Stop

The sequence of the chimeric oligonucleotide (Heme593W/X) is:



The same principle of chimeric oligonucleotide can be constructed to correct any of the mutations causing AIP depicted in Table A.

Chimeric oligonucleotides can be used to correct any other point mutation causing any of the 8 known Porphyrias in a similarly as described above.

25 Delivery of PBGD gene transfer of non viral vectors to humans

The chimeric oligonucleotide can be formulated in a vehicles preparation containing anionic or cationic phospholipids or phospholipids mixed with neutral lipids or lictosylated PEI.

30

Alternatively, the non-viral vectors can be formulated in liposomes containing mixtures of natural phospholipids and neutral lipids.

Specific protein sequences can be incorporated into liposomal membranes, that recognizes cellular receptors for specific targeting of non-viral vectors to a specific cell type such as liver, neuronal tissue or erythropoietic tissues, can be incorporated. Alternatively specific

antibodies recognizing specific cellular surface antigens can be used for targeting. Thirdly, carbohydrates on the liposomal membrane can be used for liver uptake of chimeric oligonucleotides.

- 5 The formulated chimeric oligonucleotide (HemeBiotech 595 W/X) will be administered by sc. or IV. injections to AIP patients.

The efficacy of the treatment can be evaluated as above.

- 10 Gene therapy is also as an alternative treatment of other porphyric diseases.

The gene therapy strategies outlined herein can also be used for other Porphric diseases. The general principle is to increase the cellular or systemic content of a particular defective enzyme causing the disease. The following Porphyric diseases can be encompassed by this strategy:

15

ALA deficiency porphyria (ADP)
Porphyria cutanea tarda (PCT)
Hereditary coproporphyria (HCP)
Harderoporphyria (HDP)

- 20 Variegata porphyria (VP)

Congenital erythropoietic porphyria (CEP)
Erythropoietic protoporphyria (EPP)
Hepatoerythropoietic porphyria (HEP)

- 25 In the following, preferred embodiments of the invention is primarily disclosed relating to rhPBGD

EXAMPLE 1

- 30 Fermentation of recombinant human Porphobilinogen Deaminase (rhPBGD)

Strain PBGD-1 is an *E. coli* K12 host strain JM105 genotype endA thi rpsL sbcB15 hsdR4 $\Delta(\text{lac-proAB})$ [F'traD36 proAB lacI^q $\Delta(\text{lacZ})$ M15] containing the expression plasmid pExp1-M2-BB. Strain PBGD-2 has the same expression plasmid pExp1-M2-BB but the host cell is
35 deleted for the hemC gene to facilitate rhPBGD purification. Since the strain PBGD-2 was

not ready at the start of the study, the decision was made to start the study with strain PBGD-1. Both the strains are resistant against both tetracycline and ampicillin, but due to regulatory advantages it was decided to use oxytetracycline as selection pressure. To focus the first part of the study on the expression level of rhPBGD, and not the strain stability, it was decided to start the development with selection pressure in the fermenter. When the expression level was satisfactory strain stability without selection pressure in the fermenter should be investigated. Preliminary tests performed showed that the expression level of rhPBGD was 1,5 times higher at 37°C compared to the expression at 30 °C. At 42°C it was as much as 3 times higher. Based on this knowledge one would suggest a temperature induction to either 37°C or 42°C during the fermentation to boost the rhPBGD production. However, at higher fermentation temperatures the strain stability might be a problem. The time frame was too narrow to study the rhPBGD expression at all three temperatures, so the decision was to start the study without temperature induction and to keep the temperature at 30°C during the whole process.

15

Short description of the work

During the first two months the strain PBGD-1 was cultivated on agar plates and in shake flasks to obtain information about the strain characteristics. In parallel to this purchase of study dedicated chemicals, build up of the documentation system and technology transfer of the analytical methods took place. When the PBGD-1 intermediary cell bank was prepared the actual fermentation work started. First two "simple" 1-L batch fermentations of strain PBGD-1 were used to test the newly designed substrate and to calculate the maximum growth rate for the strain. After that three 10 L fed batch fermentations of strain PBGD-1 was performed.

25

As soon as the strain PBGD-2 was available and an intermediary cell bank was prepared, this strain was implemented in the fermentation procedure developed for strain PBGD-1. At present two 10-L fermentations of strain PBGD-2 have been performed.

The general outline of the fermentation is starting with inoculum preparation on M9-tc agar plates and shake flasks. The cells are incubated at 30°C for 24 h on M9-tc agar plates and are then transferred to M9-tc shake flasks. The shake flasks are incubated for 12-14 h at 30°C. The broth from 1-2 shake flasks are used to inoculate the 10 L fermenter containing a minimal medium supplemented with yeast extract, trace elements, thiamine and oxytetracycline as selection pressure. The fermentation starts with a 14-h batch phase

where the cells grow at maximum growth rate. The glucose feed is started after 14 h and the feed rate profile is varied between 25-75 ml/h of a 600 g/l⁻¹ glucose solution.

Broth taken from shake flasks and fermentations have been used to develop the down stream processing and to test and adjust the analytical methods provided. The general outline in the down stream processing is concentration of the fermentation broth on a 0,22 µm cross flow membrane followed by diafiltration (washing) with a buffer to exchange 90-95 % of the substrate with buffer. The diafiltered cell concentrate is homogenised in a homogeniser, where the pressure has been varied between 600 – 1000 bars. The cell debris is then removed from the homogenate either by filtration on the same membrane as mentioned above or by centrifugation. Finally the extract is sterile filtered into sterile containers.

Results

15

Fermentation

The maximum growth rate for strain PBGD-1 was determined in shake flask experiments and in 1-L batch fermentations. The results are summarized in Table 5 below. The reason for the lower values in the shake flask with fermenter medium is probably acetic acid production and hence lower pH since the pH is not controlled. No experiments have been performed to calculate the maximum growth rate of strain PBGD-2, but from the fact that the batch phase has the same duration as for PBGD-1 we can draw the conclusion that the maximum growth rate is approximately the same.

25 Table 5 Maximum growth rates

Conditions	Maximum growth rate (μ_{\max}) [h ⁻¹]
M9-tc Shake flask	0,3
Shake flask with fermenter substrate	0,3
1 L Fermenter with pH controlled at 7,0	0,4

The developed substrate for the fermentation is given in Table 6 on the next page. When implementing strain PBGD-2 it seems like this strain has different requirements on either the amount of yeast extract or the thiamine concentration in the substrate. When using the substrate developed for strain PBGD-1 the growth stops or lags during the fermentation

(PD14). When adding extra yeast extract and thiamine the growth starts again. This pattern is repeated at least two times during the fermentation.

Table 6 Fermenter substrate

5

Component	Mw [g/mol]	Concentration	Unit
$(\text{NH}_4)_2\text{SO}_4$	114,12	2,70	[g/l]
KH_2PO_4	136,08	3,25	[g/l]
$\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$	228,23	2,80	[g/l]
$\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$	258,07	0,60	[g/l]
Yeast extract		5,00 - 20,0	[g/l]
$\text{C}_6\text{H}_{12}\text{O}_6 \cdot \text{H}_2\text{O}$	198,17	10,00	[g/l]
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	246,50	1,07	[g/l]
Thiamine chloride $\text{C}_{12}\text{H}_{18}\text{Cl}_2\text{N}_4\text{OS} \cdot x\text{H}_2\text{O}$		1,00 - 10,0	[mg/l]
H_3BO_3	61,83	2,1	[mg/l]
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	249,70	10,5	[mg/l]
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	270,30	35,5	[mg/l]
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	169,02	6,6	[mg/l]
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	287,50	5,3	[mg/l]
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	237,93	9,3	[mg/l]
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	147,02	14,0	[mg/l]
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	241,95	9,3	[mg/l]
HCl	34,46	6,9	[ml/l]
Oxytetracycline $\text{C}_{22}\text{H}_{24}\text{N}_2\text{O}_9 \cdot \text{HCl}$	496,90	6,0	[mg/l]

The strains seem to utilise different components in the yeast extract in a sequential order. The metabolism and respiration is different for different compounds. This gives rise to an irregular fermentation pattern with large changes in the respiration of the population during the fermentation, e.g. the CO_2 and the O_2 outlet gas analysis and the dissolved oxygen tension (DOT) signal (see Figure 12).

As the fermentation proceeds, the fermentation broth is gradually coloured bright pink. When centrifuging broth for dry weight analysis it is observed that it is the actual cells and

not the supernatant that is pink. The colonies on the M9-tc agar plates used to inoculate the shake flasks are not coloured pink, they are rather yellow or white like "normal" *E.coli* cells.

- 5 The colonies on the agar plates used for the colony forming units (CFU) analysis from the fermentation are also pink. However, on the CFU plates from PD14, the first fermentation with the new strain PBGD-2, a small portion of yellow or white colonies was observed. This observation was made already from the plates spread with broth from the inoculum shake flask. The percentage of yellow-white cells was varying in the range 2-8 % during the
- 10 fermentation. Both the white and red colonies were resistant against the antibiotic oxytetracycline. When observing the white and red colonies in the microscope they both appeared as *E.coli* rod like cells. It was hard to see any clear difference, but possibly the white cells were a little bit shorter than the red ones. To investigate this further shake flask cultivation were started with one red and one yellow colony. The CFU analysis showed that
- 15 there were only red colonies from the shake flask inoculated with the red colony, but that the white colony gave rise to approximately 70% white and 30% red cells. The rhPBGD activity and protein concentration were measured in the broth from these shake flasks. The results are shown in Table 7 below. The difference in the protein concentration and the rhPBGD activity is in accordance with the difference in the OD₆₂₀ reached in the shake
- 20 flask, probably due to different size of the inoculum colony.

Table 7 rhPBGD activity and total protein from single colony shake flasks

Start colony	Protein [mg/ml broth]	PBGD activity [U/L broth]	Specific activity [U/mg protein]
White	0,01	9	0,8
Red	0,04	27	0,7

- 25 In the Table 8 below a summary of the final values of the fermentations are given. The lower OD₆₂₀ and Dw (dry weight) values in fermentation PD12 is a result of the lower amount of glucose that totally was fed into the fermenter in this fermentation (600 ml compared to approximately 850 ml in PD11 and PD14). It is also interesting to notice the very high expression and specific activity of rhPBGD in fermentation PD14 compared to the
- 30 earlier fermentations.

Table 8 Summary of final fermentation results

Batch	Strain	Time [h]	OD ₆₂₀	Dw [g/l]	PBGD activity [U/ml broth]	Specific PBGD activity [U/mg protein]
PD11	PBGD-1	27	82	29	7,7	2,6
PD12	PBGD-1	31	59	19	15,3	1,8
PD14	PBGD-2	30	87	32	39	3,1

Until now we have achieved the best fermentation results in fermentation PD14.

- 5 In the Figures 12 and 13 the fermentation results from this fermentation with the new strain PBGD-2 are shown. After a 14-h batch phase the glucose feed is started according to a schedule with three step changes in the feed rate. However after 16 h the glucose begins to accumulate in the fermenter due to that something else is limiting the growth more. The glucose feed is then stopped and restarted when the glucose concentration becomes
- 10 limiting again.

The respiration pattern (i.e. CO₂, O₂ and DOT signals) indicated that something in the substrate was depleted after 14,5 and 22,3 h and 26,3 h (see Figure 12). When extra yeast extract and thiamine was added to the fermenter growth respiration increased dramatically

15 for a while. There was a steady increase in the OD₆₂₀ and Dw during the whole fermentation and the final values are rather high. The increase in produced amount of rhPBGD correlates very well with the increase in biomass. This is something that has been observed also in the other fermentations. However, in fermentation PD14 there also seems to be a steady increase in the specific activity of the produced rhPBGD. Something

20 that has been much less pronounced in the other fermentations.

Down stream processing

The different broths have been concentrated 1,9 – 6,9 times. The different values reflect problems with clogging of the membrane. This problem can probably be avoided by not

25 concentrating the broth too much. Instead a somewhat longer diafiltration has to be done. The homogenisation has given a good yield of released enzyme compared to sonication. Removal of cell debris is in the laboratory scale rather easily done by centrifugation. For the production scale it would be preferred to use membrane filtration and because of that filtration has been tested. However, so far the transmission of enzyme through the

30 membrane has been low resulting in low yields. This yield may be improved by better

controlled filtration parameters or extended diafiltration. Otherwise a separator could be used in the production.

In Table 9 some data from the down stream processing are shown.

5

Table 9 Summary of down stream results

Broth	Debris removal by	Sterile filtered extract			Yield from broth, %	
		protein mg/ml	activity U/ml	Spec. activity U/mg protein	protein	U
PD11	Filtration	2,7	5,1	1,9	30-60 1)	35-45 1)
PD12	Centrifugation	31	84	2,7	79	120 2)
PD14	Centrifugation	32	92	2,9	85	67
PD14	Filtration	3,8	11	2,8	15	18

1) Uncertainties in analysis, because the methods were not fully evaluated at this time.

10 2) Uncertainty in volume because of a tube leakage.

Conclusions

Strain PBGD-2 has a maximum growth rate of approximately $0,4 \text{ h}^{-1}$ in the fermenter
 15 substrate. This is similar to the maximum growth rate of strain PBGD-1, however the
 substrate requirement seems to be different for strain PBGD-2. An increase of the initial
 yeast extract and thiamine concentration in the substrate to 20 g l^{-1} and 10 mg l^{-1}
 respectively supports growth to a biomass similar to those achieved with the old strain
 PBGD-1.

20

The general fermentation process outline is a 14 h batch phase followed by a 16 h feed
 phase were the glucose feed rate is increased in three steps.

The production of rhPBGD correlates very well with the biomass production and the
 specific activity of the rhPBGD also seems to increase during the fermentation.

25 The best result so far with strain PBGD-2 is a rhPBGD concentration of 39 U/ml and a
 specific activity of $3,1 \text{ U/mg protein}$ after 30-hour fermentation. The final dry weight and
 OD_{620} was 32 g l^{-1} and 87 respectively. The plasmid stability is good during the fermentation
 when oxytetracycline is present as selection pressure.

EXAMPLE 2

Development of a purification process for recombinant human Porphobilinogen Deaminase (rhPBGD)

5

Introduction

For the capture step a weak anion exchange (DEAE-Sepharose FF) matrix has been tested primarily, because this has been the most common initial step for purification of PBGD. The disadvantage with anion exchange is that endotoxins and DNA adsorb on this
10 type of gel. There is a risk that these impurities are coeluted with rhPBGD. To use a cation exchange in this project is not possible, because pI for rhPBGD is too low. For that reason a hydrophobic gel has also been tested as a capture step.

Material and methods

15 Cell extract

Cell extract (PD12, see Example 1) was supplied frozen (8x50 ml) from Biogaia. After the initial thawing a precipitation was found in the sample. The extract was centrifuged and the next day a new precipitation was found. This means that the extract has to be centrifuged in connection to a chromatography experiment. The protein content in the extract (BCA)
20 was estimated to 29 mg/ml and the enzyme activity was found to be 63 U/ml. The pH and conductivity were estimated to 7.0 and 6 mS/cm, respectively.

Ion-Exchange

A DEAE- Sepharose FF hitrap (1 ml) column was used. The gel was equilibrated with Tris-HCl 25 mM, pH 8.5. The pH of the extract was adjusted to pH 8.5 with NaOH 5 M and the
25 sample volume applied on the gel was 1.4 or 2.0 ml. After the sample has been applied, the gel was washed with 15 column volumes with equilibration buffer. For the desorption of the gel the following KCl concentrations have been tested: 40, 120, 150 and 300 mM. Finally, after every experiment the gel was cleaned with NaOH 1 M.

30 Hydrophobic interaction chromatography

A Butyl-Sepharose 4 FF hitrap (1 ml) column was used. The gel was equilibrated with potassium phosphate 1.0-1.3 M pH 7.5. To the extract, potassium phosphate (2.5 M) was added to an end concentration of 1.0-1.3 M and the sample volume applied on the gel was 2.0 ml. After the sample has been applied, the gel was washed with 15 column volumes
35 with equilibration buffer. For the desorption of the gel 500 mM, 20 mM potassium

phosphate and water were tested. Finally, after every experiment the gel was cleaned with NaOH 1 M.

Results

5 Ion-exchange

In Figures 14 and 15 chromatograms from two DEAE runs are shown. In Table 10 the results from these runs are shown. The difference between these experiments are that peak b in the first run was desorbed with 120 mM KCl and 150 mM in the second. Further, in the first run less sample was applied and the gel was also desorbed with 300 mM KCl.

- 10 The recovery was in the best experiment found to be 75 % and the yield 47 %. To get this recovery and yield 300 mM KCl has to be used. The purity of rhPBGD in peak b (DEAE2) was estimated to 31 % (RPC).

Hydrophobic interaction chromatography

- 15 In Figure 16 a chromatogram from a Butyl run is shown. In Table 11 the result from the run is shown. In this experiment 1.3 M potassium phosphate was used and the desorption was done with water. Conductivity in peak b was found to be 60 mS/cm. The recovery was calculated to 78 % and the yield 75 %. In an investigation it was found that a precipitation was formed in the extract at a potassium phosphate concentration of 1.5 M. The purity of
20 rhPBGD in peak b was estimated to 40 % (RPC).

Comments and conclusions

- From the results of the experiments it can be seen that the mass balance in all experiments are not in balance. This seems to be valid for all analyses. The main reasons for this are
25 probably insecurity of the analyses and that all proteins are not eluted from the gel. The first reason is confirmed by the enzyme activity that seems to be too high in the extract when high concentration of potassium phosphate is added. The second reason is confirmed by the elution peak with NaOH in ion-exchange experiments. This peak is not analyzed. For the hydrophobic matrix a cleaning with organic solution can be necessary.
30 The conclusion of the results so far is that the Butyl-Sepharose 4 FF seems to be the best alternative for the capture step. The main reason for that is the higher yield of rhPBGD. Another advantage to use Butyl-Sepharose 4 FF is the small peak after cleaning with NaOH 1M compared with the large peak in DEAE-Sepharose FF runs. This probably means that few impurities stick on Butyl matrix. On the other hand there is a risk that a
35 precipitation is formed when adding potassium phosphate. A desalting before the next

chromatography step can be necessary, caused by the high ion strength in the product peak.

Table 10 Ion-exchange

5

Applied	Peak a			Peak b			Peak c		
BCA A280 Act.	BCA A280 Act.			BCA A280 Act.			BCA A280 Act.		
mg mg U	mg mg U	mg mg U	mg mg U	mg mg U	mg mg U	mg mg U	mg mg U	mg mg U	mg mg U

Exp.

10

DEAE1	37	166	76	10	122	21	5	6	29	9	11	7
DEAE2	42	229	129	17	143	56	10	14	25	-	-	-

15

Table 11 Hydrophobic interaction chromatography

20

Applied	Peak a			Peak b			Peak c		
BCA A280 Act.	BCA A280 Act.			BCA A280 Act.			BCA A280 Act.		
mg mg U	mg mg U	mg mg U	mg mg U	mg mg U	mg mg U	mg mg U	mg mg U	mg mg U	mg mg U

Exp.

25

Butyl	31	137	93	7	84	3	11	23	70	-	-	-
-------	----	-----	----	---	----	---	----	----	----	---	---	---

30 EXAMPLE 3

Development of a method for the purification of recombinant human Porphobilinogen deaminase with a "His-Tag" (rhPBGD-His)

35 Nature and purpose of the study

- Many groups have reported in the literature on the purification of porphobilinogen deaminase from various sources including *E. coli* and Human erythrocytes (Anderson P. M. and R. J. Desnick, 1979, The Journal of Biological Chemistry 255(5): 1993-99, Awan S.J. et al. 1997, Biochemistry 36(30): 9273-82, Grandchamp B. et al. 1987, Eur.J.Biochem. 162(1): 105-10, Jordan P.M. 1994, Wiley, Chichester (Ciba Found Symp 180), p70-96, Jordan P.M. et al. 1988, Biochem.J. 254:427-435, Lambert R. et al. Wiley, Chichester (Ciba Found Symp 180), p97-110, Louie G.V. et al. 1996, Proteins 25(1): 48-78, Maniatis T., E.F. Fritsch, J. Sambrook. Molecular Cloning (A laboratory Manual) Cold Spring Harbor Laboratory. 1982, Miyagi K. et al. 1979, Proc.Natl.Acad.Sci. 76(12):6172-76, Racich N. 1986, Nucleic Acids Research 14(15): 5955-67, Shoolingin-Jordan P.M. et al. 1997, Methods in Enzymology, 281:317-327). Most use a combination of ion exchange, hydrophobic interaction and size exclusion chromatography to obtain fairly pure protein preparations. With the engineering of 5 additional Histidine residues on the C-terminus of recombinant human porphobilinogen deaminase, rhPBGD we have a convenient "Tag" to help with purification. Histidine has an affinity to electropositive transition metals such as nickel, copper, zinc and cobalt. When a series of 6 or more electron-rich histidine residues are expressed on the end of a protein they can function as an anchor, firmly attaching the protein to a solid support coated with metal ions. Very thorough washing can be done without dislodging the bound moiety. Elution can be accomplished in one of two ways, either by decreasing the pH to protonate the imidazole nitrogen (pKa of 5.97) of histidine, or by including imidazole, a molecule identical to the histidine side chain, in the elution buffer which competitively dislodges the tagged protein off the support. The purpose of this study is to obtain pure rhPBGD-His for antibody production and for use as a standard in assays and protein purification.
- 25 Study objectives

The objective of this study is to obtain 10 mg of highly pure active rhPBGD-His.

Study Plan

30 Plan outline

Optimize induction time for the expression system and lysis

Purify 10 mg of rhPBGD-His for antibody production and standard

2 liter scale induction and lysis of strain

DEAE ion exchange chromatography

35 Immobilized metal affinity chromatography

Characterization of rhPBGD-His

SDS-PAGE

Amino acid analysis

Specific activity

5 HPLC

Mass spectrometry

Amino terminal sequencing

Plan body

Expression of rhPBGD-His is regulated by the bacterial Taq promoter, a derivative of the
10 lac promoter which is inducible with IPTG (See Figure 17 for plasmid map). Different
proteins are produced at different rates in *E. coli* upon induction. This necessitates the
optimization of the time required for optimum rhPBGD-His yield upon induction. To
accomplish this a culture in mid-log phase will be induced with an excess of IPTG and
expression followed at timepoints with activity and protein concentration measurements.

15 After induction the cells must be lysed to release rhPBGD-His. Of the options available,
sonication is the best for this scale of purification. It is compatible with any buffer system
and should not be damaging to the protein. To follow efficiency of lysis, absorbance at
600nm will be measured after each cycle.

For use as a standard and for antibody production at least 10 mg of rhPBGD-His will be
20 purified.

For protein purification a 2 liter flask culture of the strain producing rhPBGD-His is
sufficient. The culture will be inoculated with a fresh over-night culture of cells and grown to
mid log phase then induced with IPTG.

Plans are to utilize a two step purification process. After lysis the debris will be removed by
25 centrifugation and supernatant loaded onto a DEAE ion exchange column. This will
remove the vast majority of contaminants from the lysate and leave a limited number of
protein contaminants in the elution fractions containing rhPBGD-His. Protein will be loaded
in a high pH and low ionic strength buffer to ensure binding of the weakly charged
rhPBGD-His. Extensive washing will be used to remove material that is not firmly bound to
30 the column. A very shallow step gradient of KCl will be used to elute rhPBGD-His. This
should separate the different forms of rhPBGD-His with differing charge properties from
each other. Separation of different charged forms of PBGD, by ion exchange
chromatography, has been reported by others (Anderson P. M. and R. J. Desnick, 1979,
The Journal of Biological Chemistry 255(5): 1993-99, Jordan P.M. et al. 1988, Biochem.J.
35 254:427-435, Miyagi K. et al. 1979, Proc.Natl.Acad.Sci.USA 76(12):6172-76).

The second chromatographic step planned is a column containing Talon fast flow immobilized cobalt metal affinity resin (Clontech). This makes use of the 6-residue histidine tract at the amino terminus of the recombinant protein. Initially, a metal chelating resin (Pharmacia) charged with nickel (Sigma) was tried for purification of rhPBGD-His but it was

5 found to bind other proteins in the lysate as well which coeluted with rhPBGD-His.

Although this cobalt resin has less binding affinity to His tagged proteins than nickel it retains the high binding capacity and is more discriminating which proteins to bind. This leads to elution from the metal with a lower concentration of imidazole or with higher pH and achieves a higher level of purity. The cobalt is also bound more tightly to the matrix by a

10 tetradentate metal chelator, effectively eliminating the leaching of metal ions from the solid support during purification. The loss of reactive metal ions during elution is common problem with nickel based affinity columns (personal communications) which can lead to unwanted precipitation of purified proteins.

rhPBGD-His will be characterized by the following methods:

15 The first measure of protein purity will be by SDS-PAGE (polyacrylamide gel electrophoresis). This method will also give an indication of the molecular weight of the protein being produced.

To determine the specific activity of rhPBGD-His in the preparation it is first necessary to

20 accurately determine protein concentration in solution. Amino acid analysis will be used as an accurate method. The method also provides the amino acid composition of the protein. The concentration can be used to establish an extinction coefficient of rhPBGD-His.

Activity of the enzyme is an important measure of correct structure of the enzyme. The proper structure, equivalent to that produced in humans, is essential for rhPBGD-His to be

25 used as a therapeutic. Any deviation from the natural structure can cause activation of the patient's immune system. Historically, activity of porphobilinogen deaminase has been measured in one of two ways, either by the metabolism of porphobilinogen substrate or by the formation of preuroporphyrinogen product. In the reaction catalyzed by PBGD, porphobilinogen monomers are covalently attached one at a time starting from the free

30 alpha position of the dipyrromethane cofactor. After four molecules are added the linear tetramer of PBG, preuroporphyrinogen, is spontaneously released by hydrolysis from the cofactor, regenerating the active holoenzyme with covalently attached cofactor for further reactions (See Figure 18). After release the tetrapyrrole is circularized by the next enzyme of the heme pathway, uroporphyrinogen III synthase, forming uroporphyrinogen III, the

35 central ring of heme and vitamin B12 in animals and chlorophyll in plants. The linear

preuroporphyrinogen molecule can instead be oxidized to uroporphyrin with benzoquinone creating a molecule, which absorbs light at 405 nm. This is the basis for the activity assay used to measure PBGD activity

- The rhPBGD-His preparation will be further characterized by mass spectrometry, which will
- 5 give an accurate measure of rhPBGD-His molecular weight and potentially identify molecular heterogeneity in the preparation. rhPBGD-His can exist with 1,2,3 and 4 substrate molecules bound to it. Each substrate molecule added to the holoenzyme will add roughly 209 daltons to the mass, which is detectable through mass spectrometry. Characterization by reversed phase HPLC will provide purity data.
- 10 Amino terminal sequencing of rhPBGD-His will be used to ensure the correct amino terminus.

Materials and Methods

15 Induction and Lysis:

- From a freshly streaked colony, a culture of pExp-2 in JM105 was grown for 13.5 hours in 100 ml LB (10g/l bacto-tryptone, 5g/l bacto-yeast extract, 10 g/l NaCl pH 7.0) + 100 µg/ml ampicillin in a 500 ml baffled flask at 37°C at 350 rpm. The optical density measured at
- 20 600nm reached 1.6. This culture was used to inoculate 2 liters of terrific broth (12g/l bacto-tryptone, 24g/l bacto-yeast extract, 4ml/l glycerol, 2.31g/l KH₂PO₄, 12.54g/l K₂HPO₄ (Maniatis T., E.F. Fritsch, J. Sambrook. Molecular Cloning (A laboratory Manual) Cold Spring Harbor Laboratory. 1982) with 100 µg/ml ampicillin and split into four 2 liter baffled flasks with 400 ml each and two 1 liter baffled flasks with 200 ml culture each. These were
- 25 grown at 37°C with 350 rpm in a New Brunswick Scientific Innova 4000 incubator. When reaching an optical density of 0.7 at 600 nm the Taq promoter was induced with 4 mM IPTG, causing rhPBGD-His protein to be made. Growth was followed by hourly readings of absorbance at 600 nm. After 9 hours the cultures were stopped by chilling to 0°C after an absorbance of 1.93 was reached. The culture was centrifuged, 4 X 250ml at a time, for 10
- 30 min at 4,000xg in a Beckman Avanti J25I centrifuge with a JLA-16.250 rotor. Supernatant was decanted and the remainder of the culture was added to the cell pellets and spun for an additional 10 min. The pellets were resuspended in 2 pools of 250 ml 50 mM Tris/HCl pH 8.5 (prechilled) each and stored for 8 hours on ice. Cells were centrifuged for 10 min at 4,000xg, liquid decanted and resulting pellets weighed in the bottles to determine the wet
- 35 weights. Cells were then resuspended in 400 ml ice cold 50 mM Tris/HCl pH 8.5 and lysed

by sonication with a Branson Sonifier 450 with 1/2 inch diameter stepped, tapped horn. Each round was for 30 seconds at maximal power with constant duty cycle in a Pyrex 150 ml glass beaker on ice. Lysate was mixed between cycles by either drawing into a 50ml pipet a few times or by pouring between beakers on ice. Progress of lysis during sonication was ascertained by reading absorbance of the lysate at 600 nm. After six rounds of sonication for each of the 100 ml aliquots of cells, debris was removed by centrifuging at 16,000xg for 30 minutes at 4°C. Lysate was then pooled and vacuum filtered through a 0.22 µm Durapore membrane (Millipore) to remove any remaining particulate matter.

10 DEAE Sepharose Chromatography:

The first chromatographic step in purifying rhPBGD-His was by ion exchange chromatography on a DEAE Sepharose fast flow column (Pharmacia). A 2.5 X 50 cm Spectrum LC column with degassed resin was washed extensively with degassed 25 mM Tris/HCl pH 8.5 buffer. Filtered lysate (380 ml) was applied to the column at 5 ml/min. The column was then washed with 720 ml 25 mM Tris/HCl pH 8.5. Elution of bound rhPBGD-His was with a shallow step gradient of KCl from 50 to 120 mM in 10 mM increments in 25 mM Tris/HCl pH 8.5 and degassed. Volumes for each step varied from between 105 and 470 ml depending on the elution profile (see Table 12).

Table 12

0 mM KCl	50	60	70	80	90	100	110	120
720 ml	470	120	175	270	105	130	180	300

Fractions were collected about every 50 ml. Absorbance at 280 nm was followed closely during elution. The next step was only applied after the absorbance had declined following a peak. BioRad's protein assay II in microtiter format was used per manufacturer's protocol to assay the amount of protein in each fraction. Coomassie stained 10% acrylamide Bis/Tris gels (Novex) were then prepared, with 5 µg protein in each lane, to characterize the purity of each peak.

Cobalt Affinity Chromatography:

The resin slurry was degassed prior to pouring into a 2.5 X 30 cm Spectrum LC column. It was then washed extensively with degassed 25 mM Tris/HCl pH 8.5/ 150 mM NaCl at a flow rate of 5 ml/min. The sodium chloride was included to decrease protein to protein ionic interactions and to reduce ion exchange effects with the column matrix itself. A relatively high pH of 8.5 was used to keep rhPBGD-His well above the pI, and therefore negatively charged, to maintain high solubility during the purification. Two consecutive rhPBGD-His affinity purifications were then run on the column. The first sample loaded was a sterile filtered pool of the entire first peak of eluate of activity from the DEAE sepharose column including fractions 9 through 12. The column was then washed with 2 liters of 25mM Tris pH8.5/150mM NaCl at 3 ml/min. To elute bound contaminants the column was then washed with 100 ml of 25mM Tris pH8.5/150mM NaCl/5mM imidazole at 5 ml/min followed by 100 ml of 10mM imidazole buffer solution. Elution of his tagged protein was with 25mM Tris pH 8.5/150mM NaCl/50mM imidazole at 5 ml/min. A final elution with 100 mM imidazole was included to be certain all rhPBGD-His was eluted. To prepare the column for the second loading it was merely washed with ~100 ml of 25mM Tris pH8.5/150mM NaCl. It was hoped that rhPBGD-His would displace the imidazole bound to the column (which turned out to be the case). The second loading of the column was with a sterile filtered pool with ~900 ml of all remaining peaks of activity from the DEAE Sepharose column at a flow rate of 5 ml/min. The column was then washed with 2 liters of 25mM Tris pH8.5/150mM NaCl at 5 ml/min, followed by imidazole containing buffers as with the first run above.

Polyacrylamide Gel Electrophoresis: (SDS-PAGE)

25

Gel electrophoresis was with the Novex system with Nupage 10% Bis/Tris gels run at 125V for 2 hours with or without reducing agent. Staining was with 50% methanol / 10% acetic acid / 0.25% Coomassie brilliant blue R-250 for 2 to 4 hours. Destaining was in 30% methanol / 10% acetic acid in a Bio-Rad gel destainer.

30

Amino Acid Analysis:

Amino acid analysis was performed by AAA Laboratory (6206 89th Avenue Southeast, Mercer Island, Washington 98040). rhPBGD-His was hydrolyzed for 20 hours with 6N-HCl / 0.05% mercaptoethanol / 0.02% phenol at 115°C. Serine was increased by 10% and

35

Threonine increased by 5% to compensate for destruction of the individual acids during hydrolysis. A Beckman 7300 Amino Acid Analyzer was used coupled with System Gold software. Analysis was performed by post-column derivitization with ninhydrin using the ion-exchange chromatographic methods developed by Moore and Stein.

5

PBGD Activity Assay:

We performed assays in 96 well microtiter format with validation in cuvetts. Procedures were derived from published procedures (Awan S.J. et al. 1997, Biochemistry 36(30): 9273-82, Shoolingin-Jordan P.M. et al. 1997, Methods in Enzymology, 281:317-327). From 0.125 to 8 µg of purified rhPBGD-His protein per well have been used to determine enzymatic activity. Assay buffer is 50 mM Tris/HCl pH 8.2 with 1.0 mg/ml BSA (Sigma fraction 5) and 10 mM DTT. A Perkin Elmer 9700 PCR machine was used for thermal regulation, allowing for tight control of the temperature and reaction time. Assays have been started in two ways. One method was to start the reactions at 37°C with prewarmed substrate in a PCR block. Strategic placement of pauses in a thermocycle program was used with beeping at defined intervals for both addition of the substrate and for stopping the reaction. An example cycle program is shown in Table 13 with reaction times varying from 10, 20, 40 and 60 minutes.

20

The reaction block is a 96 well block with tubes arranged in an 8x12 matrix. It is kept throughout at 37C. The reaction is initiated by adding PBG to eight tubes in the first row using an eight-channel pipettor. The addition is staggered so that each row receives PBG every 30 seconds. A ten second pause and beep interval is setup every 20 seconds to signal each addition at the end of the period. In this fashion all the 96 reactions are started which takes a total of six minutes. At the end of a further four-minute incubation, the first three rows are stopped in a staggered manner giving a total of a ten-minute incubation period. This procedure is repeated for the next three rows after an additional ten minutes amounting to a total of twenty-minute reaction time. This scheme is illustrated in Table 13. The p@37 represents the 10-second beep period which is configured in the thermocyclor as a pause plus beep interval.

35

Table 13

start	stop	stop	stop	stop
add	10	20	40	60
PBG	min	min	min	min
<div><div><div> —12X— </div><div>37 p@37 37</div><div>20 sec 10 sec 4 min</div></div><div><div> —3X— </div><div>37 p@37 37</div><div>20 sec 10 sec 10 min</div></div><div><div> —3X— </div><div>37 p@37 37</div><div>20 sec 10 sec 20 min</div></div><div><div> —3X— </div><div>37 p@37 37</div><div>20 sec 10 sec 20 min</div></div><div><div> —3X— </div><div>37 p@37 37</div><div>20 sec 10 sec</div></div></div>				

- 5 Reactions were stopped by acidification with HCl / p-benzoquinone solution. The final concentration of HCl used was 1 molar. Benzoquinone, which oxidizes the uroporphyrinogen to uroporphyrin, was used at a final concentration of 0.002% w/v (from 0.2% stock solution in methanol). At defined intervals the 150 μ l samples were removed from the reaction tubes and added to 850 μ l HCl / p-benzoquinone solution in wells of a 96
- 10 well X 2 ml plate on ice. The second method of initiating the assay was to set up the reactions complete with substrate on ice then to transfer to the PCR block for incubation at 37°C. Following the reaction the block was brought to 4°C to stop the reaction after which samples were removed and added to HCl / p-benzoquinone solution. For both methods the incubation was allowed to proceed for 20 minutes on ice and in the dark after the last
- 15 addition of reaction solutions. Then the plate was centrifuged for 10 min at 3750 rpm in a swing out rotor in a GS-6KR centrifuge to pellet precipitated protein (mostly BSA). 250 μ l was removed to a Corning 96 well assay plate. Absorbance was measured at 405 nm with a 605 nm reference wavelength in a BioTek FL-600 plate reader. Selected samples (normally the standard curve) were diluted 10X with 1M HCl and read in a quartz cuvet in a
- 20 Beckman DU640B spectrophotometer at 405.5 nm. A 605 nm reference wavelength was used to subtract out background absorbance. These measurements in cuvetts produced a conversion factor from 1 cm pathlength reads to the plate data. Analysis was performed using the KC4 software included with the plate reader and with excel spreadsheets. An extinction coefficient of 548 M⁻¹cm⁻¹ was used to quantitate the oxidized reaction product
- 25 (Shoolingin-Jordan P.M. et al. 1997, Methods in Enzymology, 281:317-327).

HPLC:

- HPLC analysis was performed at the University of Washington Mass Spectrometry
- 30 Analysis Facility for HPLC. Samples were prepared free of salts for mass spectrometry

analysis by HPLC on a C4 column and eluted with an increasing gradient of acetonitrile. The instrument used was an Applied Biosystems (ABI) 140A Solvent Delivery System with an ABI 785A Programmable Absorbance Detector.

5 Mass Spectrometry:

- Mass spectrometric analysis was performed at the University of Washington Mass Spectrometry Analysis Facility. One tenth of the HPLC run within the main elution peak was diverted prior to the absorbance detector to a Perkin Elmer SCIEX API3 Biomolecular
- 10 Mass Analyzer for electro-spray mass spectrometry. Analysis was by HyperMass method on an average of 16 peaks (for Cobalt run #1 eluate).

Amino Terminal Sequencing:

- 15 Amino terminal sequence analysis was performed at the University of Washington Mass Spectrometry Analysis Facility. An ABI 477A Protein Sequencer was used with an ABI 120A PTH Analyzer.

Results

Purification:

20

Induction and Lysis:

Growth of the 2-liter culture of bacteria slowed down after the first hour but growth still continued to 9 hrs (see Table 14).

25

Table 14

start	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	7 hr	8 hr	9 hr
0.699	1.300	1.521	1.607	1.660	1.732	1.797	1.841	1.890	1.927

- After about 3 hrs of induction cells tended to clump together with most turbidity settling out of the broth by gravity in about an hour. Final density of cells stayed low for growth in a rich media such as terrific broth but final weight of pellets was adequate. The total wet weight was 35.3 g, corresponding to 17.7g/liter culture. Interestingly the cells were orange/pink probably due to various intermediates in the heme biosynthetic pathway. It is clear from
- 30

the low growth rate and final densities achieved that cultures were limited by the amount of oxygen available.

Lysis by sonication was essentially complete after 5 cycles as seen by following 5 absorbance readings (Table 15).

Table 15

# rounds	0	1	2	3	4	5	6	7
OD600	30.33	20.15	12.90	9.43	6.14	3.86	3.31	2.99
% down	-	34	36	27	35	37	14	9.7

10 It appears from the % decrease of optical density that for each of the first 5 rounds of sonication, about the same percentage of cells were lysed. After this the percentage of newly lysed cells dropped rapidly. For each of the first 4 rounds viscosity of the lysate was relatively high due to the presence of unfragmented genomic DNA but this decreased significantly after further rounds from shearing of the DNA into smaller fragments.

15

DEAE Sepharose:

Elution of proteins from the DEAE ion exchange column occurred in 4 distinct peaks as seen in the elution profile in Figure 19 and by protein assay in Table 16. SDS-PAGE analysis of the eluted fractions shows that these peaks contain four separate peaks of rhPBGD-His eluted with the step gradient of KCl (Figure 20). The first and major peak was eluted in fractions 9 through 13 with 50 to 70 mM KCl. As seen by gel analysis (see Figure 20) purity was fairly good for a first step of the purification, especially in fractions 10 through 13. The second peak eluted in fractions 15 through 18 with 80 mM KCl. The major contaminant in this peak, in about equal molar proportions to desired product, was a protein running at about 5 kDa smaller than rhPBGD-His. The third peak in fractions 20 through 23 eluted with 90 to 100 mM KCl and had less visible contaminants than the second peak. The fourth and final peak eluted in fractions 26 through 29+ with 110 to 120 mM KCl. The fractions were split into 2 pools for further purification. The first pool, comprising the major peak of rhPBGD-His elution contained fractions 9 through 12. The second pool contained fractions 13 through 29 along with the next 50 ml of 120 mM KCl

elution buffer. These two pools eluted by ion exchange contained 877 mg protein out of 3253 mg loaded, corresponding to a 3.7 fold decrease in total protein (See Table 16).

Cobalt Affinity:

5

From the first cobalt run the majority of rhPBGD-His eluted in a sharp peak with a volume of 30 ml upon addition of 50 mM imidazole (see Table 17 for protein assay and Figure 22 for SDS-PAGE results). A final elution with 100 mM imidazole released no detectable protein absorbing at 280nm. In the second cobalt run (Figure 21) surprisingly, the first
10 imidazole wash of 5 mM eluted a small uncolored peak of absorbance with a volume of about 50 ml. The second wash with 10 mM imidazole then eluted a larger and broader orange/pink colored peak of about 150 ml. Further elution with 50 mM imidazole yielded a large sharp uncolored peak of 23 ml.

Characterization:

15

Amino Acid Analysis:

Amino acid analysis of 3 of the fractions (Cobalt run #1 50 mM imidazole eluate (in duplicate,) Cobalt run #2 10 and 50 mM imidazole eluates) yielded conclusive data that
20 rhPBGD-His was being purified. Results from the analysis allowed for a very accurate measure of protein concentration calculated from the concentration of individual amino acids (see Table 18).

Specific Activity:

25

Specific activity of the first 50 mM imidazole eluate of rhPBGD-His from the cobalt column turns out to be high at approximately 24 U/mg (Units are in μmol PBG consumed per mg protein in one hour). Activity of rhPBGD-His was found to be strongly dependent on pH with a sharp rise from 7.0 to 8.0 where it approached a plateau. The optimum was around
30 pH 8.2. The optimum PBG substrate concentration was found to be around 1 mM. rhPBGD-His had activity with all concentrations of PBG, however with amounts less than 1 mM the reaction was limited by available substrate, decreasing both the V_{max} and the linearity over time as substrate was depleted. It was not found to be necessary to decolorize remaining benzoquinone with sodium metabisulfite as used in a published assay
35 (Shoolingin-Jordan P.M. et al. 1997, Methods in Enzymology, 281:317-327). Strangely

enough if acidification and oxidation were done in a smaller total volume (240 μ l vs 1 ml) as done by this research group then a highly colored product develops during the incubation on ice. This product must be decolorized with a saturated solution of sodium metabisulfite to obtain accurate reaction absorbances. There was no significant difference in enzymatic activity found as measured by these two variations of the method.

Generally assays have been set up with both time and enzyme concentration as variables. This allows for a more detailed analysis of the results with a built in validation. If activity is fairly linear from different timepoints at any enzyme concentration then it can be inferred that substrate is not limiting and that reaction measurements are valid over that range. If a measurement is taken at only one timepoint then there is no indication of whether the enzyme is still functioning at V-max.

Reaction volumes in 96 well format have been limited by the size of PCR tubes to 150 μ l. Volumes from between 50 and 150 μ l have been tried with a noticeable increase in linearity over time and with increasing enzyme amounts seen with the larger volumes. Additional increases in volume would make even more substrate available and dilute the protein further, thereby increasing the linearity over time and enzyme concentrations. However the increase in cost of the assay from PBG substrate would be substantial.

For routine analysis of similar protein preparations at similar concentrations it should be possible to standardize the assay and use far fewer data points and still obtain an accurate measure of PBGD activity. Optimally a standard curve of rhPBGD-His of known activity would be included to validate the results and to simplify analysis. Basically multiple variables including time would be internally controlled. With a four-parameter logistic curve of the standards one could use any time point and a wide range of sample concentrations to obtain accurate activity measurements. Single use aliquots of highly pure rhPBGD-His could be stored frozen for use as standards.

30 Mass Spectrometry:

Mass spectrometry of the 50 mM imidazole elution peaks from the two cobalt runs yielded molecular weights of:

- 1st cobalt run eluate: 38,816.8, standard deviation = 3.68
35 2nd cobalt run eluate: 38,814.6, standard deviation = 4.70

1st cobalt eluate dialyzed for antibodies: 38,817.1, STD deviation = 3.33

These weights correspond to the holoenzyme without any additional substrate molecules attached.

5

Evaluation and conclusions

We found that with a simple two step purification process involving ion exchange and cobalt affinity chromatography we could achieve a yield of 173 mg/l rhPBGD-His with a
10 purity of greater than 98% starting from a bacterial crude lysate. Each one of the enzyme intermediate complexes is stable and can be independently isolated (Anderson P. M. and R. J. Desnick, 1979, The Journal of Biological Chemistry 255(5): 1993-99, Jordan P.M. et al. 1988, Biochem.J. 254:427-435, Miyagi K. et al. 1979, Proc.Natl.Acad.Sci.USA 76(12):6172-76). This may be a major contributing factor to the differential binding of
15 different enzyme fractions to the DEAE ion exchange matrix. Due to the negative charges contributed by acetate and propionate side groups on the growing chain of porphobilinogen molecules it could be theorized that binding affinity to the ion exchange resin would be in the order; E<ES<ES2<ES3. That would imply that the first peak could be the holoenzyme followed by the others in the same order of the reaction progression. The cobalt column
20 also eluted rhPBGD-His in different fractions during the second run. It is strange that the elution profile from the second run was different from the first. It would be expected that all closely related proteins with a his-tag would bind to cobalt with the same affinity. This implies that either the His-tag is partially digested away or partially obscured due to protein conformational changes or charge interactions. The difference in elution characteristics
25 may also be due to differences between the various enzyme-substrate intermediate complexes as hinted by the color difference in the 10 mM elution peak. From a report in the literature by Jordan P.M. 1994, Wiley, Chichester (Ciba Found Symp 180), p70-96, the ES2 intermediate complex has a pink colored chromophore. The 10 mM imidazole fraction from the second cobalt column run has a pink color while the other fractions do not. This
30 implies a separation of different enzyme substrate intermediates in different fractions. If the colored protein peak is predominantly composed of the ES2 intermediate then it could be extrapolated that the peak at 5 mM would be ES3. Whatever would be decreasing the binding of ES2 to cobalt whether conformational or charge related as compared to ES would likely be enhanced with the ES3 intermediate. The peak released with 50 mM
35 imidazole and with stronger binding to DEAE could then be the ES form. Holoenzyme by

itself may be the remaining form, purified during the first cobalt run, binding less tightly to DEAE due to a higher pI and elute from nickel with 50 mM imidazole. When the mass of the two 50 mM cobalt eluates were compared however there was no significant difference detected. Both corresponded to the weight expected for holoenzyme alone. Unfortunately

5 no reliable mass measurement of the 10 mM eluate was obtained due possibly to precipitation problems with a lower rhPBGD-His protein concentration. If a difference of elution characteristics between the different enzyme substrate intermediates is occurring then a likely explanation would be due to the large conformational changes that take place during the course of the reactions From E to ES4 (Jordan P.M. 1994, Wiley, Chichester

10 (Ciba Found Symp 180), p70-96, Louie G.V. et al. 1996, Proteins 25(1): 48-78). The C-terminal His-tag on the third domain of the protein could become partially hidden and rendered sterically less accessible when the reaction proceeds past the ES1 form. A direct interaction between the his-tag and the growing substrate chain would be less likely. At a pH of 8.5 histidines should be in an electron rich unprotonated state and the substrate

15 complex should also be in an electron rich state even though acidic side chains are neutralized by basic amino acids in the catalytic cleft (Jordan P.M. 1994, Wiley, Chichester (Ciba Found Symp 180), p70-96, Louie G.V. et al. 1996, Proteins 25(1): 48-78).

Conformational changes in rhPBGD-His occurring during the reaction could conceivably make accessible other charge groups for interaction with the his-tag either on the surface

20 or perhaps the same ones meant for dampening charges from the growing substrate polymer in the cleft.

Equipment and supplies lists are shown in appendix 4 and 5, respectively.

Appendix 4 Equipment list:

Item	Manufacturer	Serial Number
Pipetman P-1000	Gilson	N55287E
Pipetman P-200	Gilson	N52324E
Pipetman P-20	Gilson	N53465M
Pipetman P-10	Gilson	P626586
5415C centrifuge	Eppendorf	5415B68381
GS-6KR centrifuge	Beckman	NGD97J18
Avanti J-25 I centrifuge	Beckman	JJY97J14
DU 640B Spectrophotometer	Beckman	4323015
Genie II vortex	VWR	2-241186

GeneAmp PCR system 2400	Perkin Elmer (PE) / Applied Biosystems (ABI)	803N6021903
GeneAmp PCR system 2400	PE / ABI	803S7100104
GeneAmp PCR system 9700	PE / ABI	805S7121566
1545 incubator	VWR	0902597
BioTek FL-600 plate reader	BioTek	
ProTeam LC System 210	ISCO	
Nupage Electrophoresis System	NOVEX	
Gel Destainer	BioRad	
Power Pac 200	BioRad	
Power Pac 1000	BioRad	
Innova 4000 incubator	New Brunswick Scientific	890165366
Innova 4000 incubator	New Brunswick Scientific	
Power Mac G3 computer	Macintosh	XA8061A3BBW
Trinitron Multiscan 200GS monitor	Sony	8057052
DNA analysis Software: Geneworks	Intelligenetics	Version 2.5.1
Sonifier 450	Branson	
1/2" diameter stepped disruptor horn	Branson	
2.5 X 50 cm LC column	Spectrum	
1.5 X 30 cm LC column	Spectrum	

Appendix 5 Supplies List

Item	Supplier	Cat #	Lot #
Ampicillin	Sigma	A-9518	76H0434
Bacto Agar	Difco	0140-07-4	106728JA
Tris six-pack "C"	Sigma	T-PAC-C	77H9049
Trizma Base	Sigma	T-8524	28H5436
HCl	Sigma	H-1758	37H3495
PBG (5mg)	Sigma	P-1134	77H0930
PBG (1mg)	Sigma	P-1134	36H1297
BSA (fraction 5)	Sigma	A-6003	87H7603
DTT	Sigma	D-9779	105H7711

Methanol	Fisher	A452SK-4	982215
P-Benzoquinone	Acros	10563-0050	A011202801
DEAE Sepharose Fast Flow	Pharmacia	17-0709-01	256288
Chelating Sepharose Fast Flow	Pharmacia	17-0575-01	253865
Nickel Sulfate	Sigma	N73-100	985482
Talon Superflow Metal Affinity Resin	Clontech	8908-2	8110601
Centricon Plus-80 (Biomax 8)	Millipore	UFC5 BFC 02	Not available
Nupage 10% Bis/Tris gels	Novex	NP0302	Various
Protein Assay Kit II	BioRad	500-0002	59163A,62171A
Centricon-10	Millipore	4321	L8PM2042
KCl	Sigma	P-9333	68H01001
NaCl	Sigma	S-3014	97H1151
Imidazole	Fisher	03196-500	985421
Corning microtiter plate	Fisher	07-200-89	Not available
Costar 96 well X 2ml plate	Fisher	097-61-117	Not available
MicroAmp Reaction Tubes	Perkin Elmer	N801-0838	S18N8-41
MicroAmp Full Plate Cover	Perkin Elmer	N801-0550	090397
Spectra/Por 2.1 Biotech DispoDialyzers MWCO: 15k	Spectrum	135030	11987

Table 16 Protein assay results on DEAE fractions (with BioRad's Protein assay II kit):

sample	vol(ml)	mM KCl	protein rhPBGD-His			gel #	well #	protein	
			mg/ml	mg/ml	mg prot			pool	mg pool
DEAE Load	380	0	8.56		3253	1	2		
DEAE FT	380	0	1.33		505	1	3		
DEAE #1	16	0	0.26	0.00	4	1	4		
2	60	0	0.23	0.00	14	1	5		
3	100	0	0.044	0.00	4	1	6		
4	215	0	0.002	0.00	0	1	7		
5	320	0	0.005	0.00	2	1	8		
6	75	50	0.11	0.00	8	1	9		
7	100	50	0.19	0.00	19	1	10		
8	100	50	0.14	0.00	14	1	11		
9	94	50	0.61	0.31	57	1	12	1	334
10	100	50	1.44	1.15	144	2	1	1	
11	38	60	1.05	0.84	40	2	2	1	
12	135	60-70	0.69	0.48	93	2	3	1	V
13	100	70	0.4	0.10	40	2	4	2	543
14	50	80	0.43	0.09	22	2	5	2	
15	50	80	0.96	0.34	48	2	6	2	
16	50	80	0.98	0.49	49	2	7	2	
17	50	80	0.57	0.29	29	2	8	2	
18	50	80-90	0.48	0.14	24	2	9	2	
19	50	90	0.42	0.13	21	2	10	2	
20	50	90	0.61	0.24	31	3	1	2	
21	50	90-100	0.93	0.70	47	3	2	2	
22	50	100	1.08	0.86	54	3	3	2	
23	50	100	0.57	0.34	29	3	4	2	
24	50	110	0.41	0.08	21	3	5	2	
25	50	110	0.61	0.09	31	3	6	2	
26	50	110	0.69	0.17	35	3	7	2	
27	50	110-120	0.73	0.44	37	3	8	2	
28	28	120	1.07	0.86	30	3	9	2	
29	50	120	1.08	0.76	54	3	10	2	V

Table 17 Second cobalt run fractions with other samples in Figure 20 gel

Sample description	Imidazole mM	volume ml	Conc. mg/ml	Total mg	Gel lane	Ico-pure mg
Co-1 load	0	367	0,78	286,3	2	193,8
Co-1 FT	0	367	0,22	80,7	3	
Co-1 Eluate	50	30	6,46	193,8	4	
MI #1 Nickel	500 ?		9,65		5	101,5
PBGD-1 Lys	0		1,73		6	
Ab prep #1	0	3,3	4,00	13,2	7	
Cobalt-2 FT	0	900	0,23	207,0	8	
FT tail	0	30	0,02	0,6	-	
Cobalt-2 w1	5	100	0,01	1,0	-	
" w2	5	50	0,14	7,0	-	
" w3	5	52	0,02	1,0	-	
" w4	5	100	0,04	4,0	-	
" w5	10	50	0,07	3,5	-	
" w6	10	50	0,81	40,5	9	
" w7	10	50	0,81	40,5	10	51,5
" w8	10	50	0,41	20,5	11	
" w9	10	130	0,20	26,0	-	51,5
" E1	50	22,5	2,29	51,5	12	
" E2	50	30	0,11	3,3	-	

Total mg highly pure rhPBGD-His (by amino acid analysis) = **346,8**

Table 18 rhPBGD-His amino acid analysis:

5

Cobalt eluates											
aa	#aa	% whole	#1		#2		#3		#4		
			umol/ml						→		
			aa	[PBGD]	aa	[PBGD]	aa	[PBGD]	aa	[PBGD]	
Ala	29	8,31	4,8220	0,166	0,5920	0,020	1,6950	0,058	4,5406	0,157	
Arg	21	6,02	3,7279	0,178	0,4550	0,022	1,3050	0,062	3,5129	0,167	
Asn	10	2,87									
Asp	19	5,44	4,7157	0,163	0,5755	0,020	1,6510	0,056	4,4442	0,153	
Cys	4	1,15									
Gln	19	5,44									
Glu	21	6,02	6,7648	0,169	0,8152	0,020	2,3592	0,059	6,3524	0,159	
Gly	27	7,74	4,3933	0,163	0,6120	0,023	1,5595	0,058	4,1137	0,152	
His	18	5,16	2,5664	0,143	0,2539	0,014	0,7947	0,044	2,4518	0,136	
Ile	20	5,73	3,0511	0,153	0,3661	0,018	1,0561	0,053	2,8381	0,142	
Leu	43	12,32	7,0235	0,163	0,8565	0,020	2,4506	0,057	6,6020	0,154	
Lys	18	5,16	2,9241	0,162	0,3538	0,020	1,0427	0,058	2,7135	0,151	
Met	6	1,72	0,8691	0,145	0,0996	0,017	0,3072	0,051	0,8252	0,138	
Phe	9	2,58	1,4713	0,163	0,1825	0,020	0,5216	0,058	1,4045	0,156	
Pro	16	4,58	2,7268	0,170	0,3708	0,023	1,1194	0,070	2,8441	0,178	
Ser	18	5,16	2,8356	0,158	0,3570	0,020	1,0121	0,056	2,8680	0,159	
Thr	20	5,73	3,4426	0,172	0,4272	0,021	1,2154	0,061	3,2746	0,164	
Trp	2	0,57									
Tyr	3	0,86	0,5150	0,172	0,0626	0,021	0,1734	0,058	0,4823	0,161	
Val	26	7,45	4,0526	0,156	0,4920	0,019	1,4292	0,055	3,7740	0,145	
Avg umol/mol rhPBGD-His: (w/o bold values)				0,167			0,021			0,059	0,159
Avg mg/ml rhPBGD-His: (MW= 38759,4)				6,46			0,81			2,29	6,17
Volume (ml):				30			100			22,5	5
amount in fraction (mg):				193,7			80,8			51,6	30,8

sample	run #	imidazole	Notes
#1	1	50 mM	Major peak of cobalt eluate (from 50->60mM KCl elution from DEAE)
#2	2	10 mM	70->120mM KCl elution from DEAE ; cobalt fractions W-6,7
#3	2	50 mM	" fraction E-1
#4	1	-	#1 dialyzed -> PBS+ 5mM Tris/Cl pH 8.0 (used for 2nd round Antibodies)

Analysis done at:

AAA Laboratory

By:

Lowell Ericsson, Nancy Ericsson

Address:

6206 89th Ave SE, Mercer Island, Washington 98040-4599

COMBINATION THERAPY

Combination therapy of rhALAD AND rhPBGD

The etiology behind AIP is not fully understood. However, the accumulation of the two heme- precursors delta-aminolevulinic acid (ALA) and porphobilinogen (PBG) are likely to be involved. ALA and PBG have been suggested to be toxic to the central and peripheral nervous system causing the well known symptoms such as abdominal pain, muscle weakness, loss of sensory functions as well as epileptic seizures, respiratory paralysis, hallucinations and psychosis, observed during acute attacks.

10

The rationale for the enzyme substitution therapy in AIP patients is based on the administration of rhPBGD by sc. injections to lower serum and intracellular PBG levels. PBG will be metabolized to preuroporphyrinogen. Preuroporphyrinogen will subsequently enter the normal heme biosynthetic pathway and be metabolized to heme.

15 Hence, rhPBGD enzyme replacement therapy will have a dual action,
i) reduce circulating levels of toxic PBG and ii) restore heme production.

In the etiology of the disease it has been suggested that ALA might have an even more toxic effect than PBG. Therefore, a reduction of both ALA and PBG may be desired.

20 Treatment of AIP patients with rhPBGD will i) reduce circulating levels of PBG as well as ALA, since ALA and PBG are in equilibrium with each other through coupled enzyme reactions e.g. delta-aminolevulinic acid dehydratase (ALAD) and porphobilinogen deaminase PBGD and ii) restore heme production. A block in the PBGD enzyme will result in the accumulation of both PBG and ALA. Administration of rhPBGD will quickly
25 metabolise PBG and lower ALA levels as well, through changes in the equilibrium of the ALAD enzyme reaction.

An accelerated reduction of ALA might be beneficial to AIP patients. Hence, a coadministration of both rhPBGD and rhALAD will rapidly reduce both heme precursors.

30 The mixing and administration of rhALAD and rhPBGD could be done in two ways, either: i) a product containing both enzymes at fixed proportions or ii) administration of rhPBGD and rhALAD by two separate subcutaneous injections. In the latter case the dose of the two enzymes could be adjusted to obtain optimal individual therapy. Administration of separate enzymes provides also a possibility for optimal temporal order of administration to obtain
35 the best individual therapeutic effect.

The above combination of PBGD and ALAD is also believed to be beneficial in connection with treatment of HCP and VP as these patients may also have elevated levels of the enzymes PBG and ALA.

5

Combination therapy of rhPBGD and rhUroporphyrinogen III cosynthetase

Coadministration of rhPBGD and rhUroporphyrinogen III cosynthetase to some AIP patients are likely to be beneficial, by improving conversion of preuroporphyrinogen to its uroporphyrinogen III isomer rather than the I isomer. The I isomer forms spontaneously from preuroporphyrinogen and can not be further metabolised into heme. Hence, a
10 coadministration of rhPBGD and Uroporphyrinogen III cosynthetase will ensure a better restoration of normal heme synthesis in that less amount of the uroporphyrinogen I isomer will be formed.

15 Combination therapy of rhALAD, rhPBGD and rhUroporphyrinogen III cosynthetase

rhPBGDcosynthetase can be coadministered with both rhPBGD and rhALAD to specific patients to obtain beneficial heme synthesis restoration.

It is within the scope of the present invention to extend a combination therapy to other
20 enzymes mentioned herein and to treatment of the other porphyrias.

Example 5

Treatment of other porphyrias

25

In analogy with the new treatment of AIP patients with (recombinant) PBGD, hepatic Porphyrias such as ALA deficiency Porphyria (ADP), Porphyria cutanea tarda (PCT), Hereditary Coproporphyria (HCP) and Variegata Porphyria (VP) can benefit from substitution therapy by rhALA dehydratase, rhUroporphyrinogen decarboxylase,
30 rhCoproporphyrinogen oxidase and rhProtoporphyrinogen oxidase, respectively.

Patients having Erythropoietic Porphyrias such as Congenital erythropoietic Porphyria (CEP) or Erythropoietic protoporphyria (EPP) will benefit from substitution therapy with rhUroporphyrinogen III synthetase and rhFerrochelatase, respectively.

35

Hepatoerythropoietic Porphyrias e.g. Hepatoerythropoietic Porphyrias (HEP) can be treated with rhUroporphyrinogen decarboxylase.

All porphyrias can be treated by the administration of the enzymatic activity lacking or
5 being reduced (normally 50%) in any of the eight steps in the heme biosynthetic pathway as described above.

The substitution of the enzymatic activity can be achieved by adding the corresponding recombinant enzyme or other molecules that will provide the missing enzymatic activity. In
10 situations where a combination of enzymes are beneficial, such therapy may be applied in a manner similar as disclosed above.

Example 6

**Expression of housekeeping porphobilinogen deaminase from mouse in HeLa cells and
15 NIH 3T3 cells**

Experimental Procedures

Construction of the Recombinant Plasmid pNGVL3-GTC1-PBGD

A liver from a healthy mouse was homogenized and the total RNA was extracted.

Complementary DNA was synthesized from total RNA using reverse transcriptase from
20 murine leukemia virus and random priming (First-Strand cDNA Synthesis Kit, Amersham Pharmacia Biotech). The PBGD cDNA housekeeping form was amplified by using nested primers in the polymerase chain reaction (PCR). In the first primer pair the forward primer was 5'-GGAGTCATGTCCGGTAACG-3' and the backward 5'-
CAGACCAGTTAGCGCACATC-3'. In the second primer pair the forward primer was 5'-
25 CGCGGGGTCGACGCCACCATGTCCGGTAACGGCGGC-3' that contained the restriction site *SalI* and a Kozak site necessary for optimal translation and the backward primer was 5'-CCCGGGGGTACCTTAGCGCACATCATTAAG-3' that contained a *KpnI* restriction site. The amplified PBGD was digested by *SalI* and *KpnI* and ligated into the plasmid pNGVL3-GTC1 that was digested with the same restriction enzymes. The vector pNGVL3-GTC1
30 contains a cytomegalovirus (CMV) promoter and a kanamycin resistance gene obtained from National Gene Vector Laboratory (University of Michigan). *Escherichia coli* was transformed by the recombinant vector and the transformed bacteria was selected by the antibiotic kanamycin. The recombinant plasmid, pNGVL3-GTC1-PBGD, was isolated from

selected clones and the PBGD cDNA insert was confirmed by restriction enzyme analysis and sequencing.

Cell Cultures

- 5 HeLa cells and NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and gentamycin to a final concentration of 50 ng/mL.

Transfection

- Before transfection, the cells were seeded on a 3 cm 6-well plate at 100 000 cells/well and
10 grown 24 hours in an incubator at +37°C containing 5% CO₂ to a 60-70 % confluency. The cells in each well were transfected with the following polyethylenimine (PEI) based transfection protocol (final volume 20 µL):

- 5 µL of 1 µg/µL plasmid (pNGVL3-GTC1-PBGD, housekeeping form, or pNGVL3-GTC1)
- 15 • 7.6 µL H₂O
- 5 µL of 20% glucose
- 2.4 µL of a 0.1 mol/L 25 kD PEI solution

- All reagents were added to a testtube in the order described above, mixed thoroughly and left at room temperature for ten minutes. A volume of 200 µL DMEM from the well was
20 removed and mixed with the 20 µL of the transfection mixture and transferred back to the well. 24 hours after the transfection the cells were washed once with phosphate buffered saline (PBS) and lysed with 500 µL of passive lysis buffer (PLB)/well (PLB is obtained from Promega) and the plate was placed on an orbital shaker for 15 minutes. The cell lysates were kept in the freezer -20 °C.

25 PBGD Activity Assay

- The PBGD activity assay is according to Magnussen et al, Blood, 44 , 857-868, 1974 with some modifications. Three hundred µL of the cell lysate was mixed with 1.15 mL Tris-HCl buffer (50 mmol/L, pH 8.2). The reaction was initiated by adding 50 µL of 3 mmol/L porphobilinogen (PBG). The mixture was incubated at +37°C for 60 minutes in the dark in a
30 waterbath with shaking and the reaction was terminated by adding 1.50 mL of 25 % trichloroacetic acid (TCA). The samples were centrifuged for ten minutes in a Heraeus Megafuge at 3509 g to remove particles and precipitated proteins and the supernatant was transferred to a clean testtube. The samples were kept dark for 2.5 hours and the

fluorescence of the product uroporphyrin I in the supernatant was measured at the excitation wavelength 405 μm and the emission wavelength 596 μm .

A tissue blank was included in the assay, which was cell lysate from HeLa cells. A volume of 300 μL cell lysate was mixed with 1.15 mL Tris-HCl buffer (50 mmol/L, pH 8.2), and by adding 1.5 mL of 25 % TCA to the sample prior to incubation the PBGD were precipitated and no formation of product could occur.

Uroporphyrin I was used as standard with a concentration range between 0 to 33.3 ng/mL. As positive control bloodsamples from two different acute intermittent (AIP) patients was used, one with very high level of PBGD activity (erythroid form) in the erythrocytes and one with low level, and a third control was the PBGD activity in erythrocytes from a normal healthy individual.

Protein Assay

The protein concentration in the cell lysate was determined by the dye-binding reaction, Bio-Rad DC Protein Assay. Bovine serum albumin was used as standard and Seronorm™ Protein as control.

Results

The basal activity in the two different cell lines were measured (four times in HeLa and five times in NIH 3T3). Three samples from each cell line were transfected with pNGVL3-GTC1-PBGD and one sample from each was transfected with the plasmid pNGVL3-GTC1 without PBGD insert. Table 19 illustrates the results obtained from the PBGD activity analysis and the protein assay.

Table 19: Results from the PBGD activity assay and the protein assay in HeLa cells and NIH 3T3 cells, non-transfected and transfected with pNGVL3-GTC1-PBGD and pNGVL3-GTC1.

1.1.1.1.1 Sample	Protein concentration g/L	PBGD Activity (nkat/L)	PBGD Activity/Protein conc (pkat/g prot)
HeLa	2,11	0,0353	16,73
HeLa	1,88	0,0273	14,52
HeLa	1,94	0,0246	12,68
HeLa	1,54	0,0227	14,74
HeLa + pNGVL3-GTC1-PBGD	1,73	12,07	6977
HeLa + pNGVL3-GTC1-PBGD	1,94	7,858	4051
HeLa + pNGVL3-GTC1-PBGD	1,91	12,65	6623

HeLa + pNGVL3-GTC1	2,03	0,0175	8,62
NIH 3T3	1,94	0,0451	23,25
NIH 3T3	2,06	0,0494	23,98
NIH 3T3	1,76	0,0496	28,18
NIH 3T3	1,95	0,0453	23,23
NIH 3T3	1,63	0,0429	26,32
NIH 3T3 + pNGVL3-GTC1-PBGD	1,76	0,486	276,1
NIH 3T3 + pNGVL3-GTC1-PBGD	1,81	0,462	255,2
NIH 3T3 + pNGVL3-GTC1-PBGD	1,86	0,381	204,8
NIH 3T3 + pNGVL3-GTC1	1,93	0,0261	13,52

(1 nkat/L corresponds to 0.060 units/L)

Figure 23 and 24 illustrate these numbers in diagrams. The expression of PBGD in HeLa cells was increased up to 475 times from the basal activity and in NIH 3T3 cells up to 11 times. According to the method described herein, the expression of PBGD can be increased at least 100 times from the basal activity, such as at least 200 times, preferably 300 times, more preferably 400 times, such as about 500 times even at a higher increase may be obtained.

In NIH 3T3 cells an increase of at least 3 times, such as at least 6 times, preferably at least 10 times such as 11 times can be obtained according to the present method and it is believed that it may be increased further.

Example 7

P-9808: Summary and method of a high scale fermentation process using *E.coli* and a down stream process for production of extract containing active recombinant human Porphobilinogen Deaminase (rhPBGD)

5

The most important facts about the process is summarised in table 20 below.

Table 20

Step	Methods for Fermentation process
1. Strain propagation on agar plates.	4 pcs. of M9H-Tc (6 mg l ⁻¹) agar plates are inoculated with cells from a Working Cell Bank (WCB) cryo vial stored at < -70 °C. The agar plates are incubated upside down for 24 ± 4 h at 30 ± 1 °C
2. Shake flask cultivation	Two 1 L baffled shake flasks containing 250 ml M9H-Tc (6 mg l ⁻¹) substrate are incubated with growth from 1 ½ M9H-Tc (6 mg l ⁻¹) agar plate each. The shake flasks are incubated in a rotary shaker at 135 ± 15 rpm for 12-14 h at 30 ± 1 °C. After incubation the broth from both shake flasks is pooled together in a container suitable for transfer to the 14 L inoculum fermenter.
3. Inoculum fermentation	The 14 L fermenter containing 8 ± 0,4 L MM5Y-Tc (6 mg l ⁻¹) substrate is inoculated with 500 ml broth both shake flasks. The inoculum fermentation is performed as a batch fermentation with the conditions described below. When the OD ₆₂₀ is in the interval 7-10 the broth is transferred to a container suitable for transfer of the broth to the 1500 L fermenter. Fermenter: 14 L Chemap fermenter with standard type configuration Temperature: controlled at 30 ± 1 °C during fermentation. pH : controlled at 7,0 ± 0,2 by titration with 12,5% (w/v) NH ₃ and 2 M H ₂ SO ₄ pO ₂ : controlled > 20 % by manual increases in stirrer speed or aeration.

	<p>Initial stirrer speed: 800 + 100 rpm</p> <p>Initial aeration: 4,5 Nlmin⁻¹ (0,5 VVM)</p> <p>Fermentation time: Approximately 9 h (OD₆₂₀ is in the interval 7-10)</p>
4. Production fermentation	<p>The 1500 L fermenter containing 850 ± 100 L MM20Y-Tc substrate is inoculated with the broth from the 14 L inoculum fermenter. Fermentation conditions are given below.</p> <p>Fermenter: 1500 L Chemap fermenter with standard type configuration</p> <p>Temperature: Controlled at 30 ± 1 °C, when fermentation is stopped the broth is cooled down to 20-25 °C.</p> <p>pH : Controlled at 7,0 ± 0,2 by titration with 25 % (w/v) NH₃ and 2 M H₂SO₄</p> <p>pO₂: Controlled > 20 % by manual increases in stirrer speed or aeration.</p> <p>Initial stirrer speed: 200 ± 50 rpm, max 400 rpm.</p> <p>Initial aeration: 425 ± 100 Nlmin⁻¹ (0,5 VVM), max 775 Nlmin⁻¹</p> <p>CO₂ in outlet gas: Controlled < 7 % by manual increases in aeration.</p> <p>Glucose feed (600 gl⁻¹): Initiated when glucose concentration < 0,5 gl⁻¹</p> <p style="text-align: right;">0-7 h after feed start 3,0 lmin⁻¹ 7-14 h after feed start 6,0 lmin⁻¹ 14 h - End fermentation 9,0 lmin⁻¹</p> <p>2 M MgSO₄ x 7 H₂O feed: Constant feed rate of 490 mlh⁻¹ initiated at the same time as the glucose feed</p> <p>Fermentation time : Fermentation is stopped when OD₆₂₀ = 100 ± 20 (≈ 28-30 h.)</p>

Step	Methods for Down stream process
5. Cell concentration using membrane filtration	<p>300 liter broth is concentrated 2 times with a cross-flow membrane at a temperature of 15-25°C. After concentration cells are washed with a volume of 2 times the cell concentrate volume. The buffer used is 50 mM sodium-phosphate buffer + 1,34 mM EDTA, pH 7,4.</p> <p>Filter equipment: Biomax 1000 K, Millipor, Pellicon, 4 x 2 m²</p> <p>During filtration temperature, pressure and permeate-flux are controlled.</p> <p>End volume: 150 ± 10 liter.</p> <p>Permeate-flux: 15 ± 2 l m⁻²h⁻¹.</p>
6. Homogenisation	<p>Cell concentrate is homogenized 800 bar, 3 passages with a flow-rate of 100 ± 10 l h⁻¹. Homogenate temperature is set at 15-25°C between passages. After the third passage homogenate is diluted 2,5 times with 50 mM sodium-phosphate buffer+1,34 mM EDTA, pH 7,4. During homogenization temperature, pressure and permeate-flux are controlled.</p> <p>Homogenizer: Rannie Type LAB 10-51VH, Max pressure 1500 bar. Supplier APV</p>
7. Cell debris removal by membrane filtration	<p>Diluted homogenate is concentrated 2,5 times with a cross-flow membrane at a controlled temperature between 15 and 25°C. The concentrate is then dia filtered with the same membrane to a theoretical yield of 85 ± 5 % with 50 mM sodium-phosphate buffer + 1,34 mM EDTA, pH 7,4.</p> <p>Permeate (Extract) is gently stirred in a tank with nitrogen flushed over the surface. During filtration temperature, pressure and permeate-flux are controlled.</p> <p>Filter equipment: Biomax 1000 K, Millipor, Pellicon, 4 x 2 m²</p> <p>End volume permeate: 450 ± 100 liter.</p> <p>Permeate-flux: 12 ± 2 l m⁻²h⁻¹.</p>
8. Final filtration	<p>Permeate from Cell debris removal is filtered through a 0,22 µm filter into 20 liter containers. Each container is filled to a weight of 10 kg. The filter is integrity tested according to specified instructions. Filled containers are transported to a</p>

	<p>freeze-house according to instructions. Maximum time from packaging to delivery at the freeze-house is 6 hours. Extract is kept at the freeze-house until it is released for delivery to BioInvent where the final Down-stream process take place.</p> <p>End volume filtered extract: 350 ± 100 liter.</p> <p>Filter: Durapore CVGL71TP3</p> <p>Containers: 20 liter Flex-Boy</p> <p>Temperature freeze-house: <18°C</p>
--	---

METHOD OF PURIFICATION OF rhPBGD

5 INTRODUCTION

The scope of this document is to give an overview of the current method of purification of crude rhPBGD.

- 10 Cell extract is supplied by BioGaia AB frozen in 20 liter Flexboy bags, filled to 10 liters. After thawing, purification is achieved i three steps commencing with a HIC step on Streamline Phenyl FF Sepharose, followed by a IEC step on DEAE FF Sepharose, ending with an affinity step on Cibacrone Blue FF Sepharose.
- The main fraction of the affinity purification step is diafiltrated with the formulation buffer
- 15 before freezing.

STEP 1: HIC, STREAMLINE PHENYL FF SEPHAROSE

A Pharmacia BPG-200 column is packed with gel to a volume of 10,8 liters (h=310 mm, 20 diam. 200 mm). Flow is 0,5 L/min.

Crude extract (e.g. 50-100 liters) is thawed overnight in room temperature, then diluted immediately before loading onto column, with approximately 20 – 45 liters of 2,5 M $K_2HPO_4(aq)$, pH 7,5.

- 25 Column is equilibrated with 0,75 M $K_2HPO_4(aq)$, pH 7,5. Following sample loading and washing with 0,75 M $K_2HPO_4(aq)$, pH 7,5, elution is performed using water.

The main fraction (23-28 liters, pH 7,2-7,8, cond. 15-20 mS/cm) is stored in a coldroom overnight.

Chromatography sequence

No.	Action	Buffer	Col. vol.
1.	Eqvibration	0,75 M $K_2HPO_4(aq)$, pH 7,5	1
2.	Sample load	Sample	Ca. 12
3.	Wash	0,75 M $K_2HPO_4(aq)$, pH 7,5	3
4.	Elution	Water	5

5

STEP 2: IEC, DEAE FF SEPHAROSE

A Pharmacia BPG-300 column is packed with gel to a volume of 12,5 liters (h=180 mm, diam. 300 mm). Flow is 0,5 L/min.

10

Main fraction from step 1 is diluted with water to a conductivity of 4-5 mS/cm (4-6 volumes water) and split into two equal halves. Step 2 is run twice following each other as follows: The column is equilibrated with 10 mM $K_2HPO_4(aq)$, pH 7,5. The sample is loaded onto the column. Washing is performed first with 10 mM $K_2HPO_4(aq)$, pH 7,5 (about 2 col. vol.), followed by 10 mM $K_2HPO_4(aq)$, 6 M urea, pH 7,5 (about 2 col. vol. for the purpose of removing ECP). Elution is performed with 10 mM $K_2HPO_4(aq)$, 100 mM KCl(aq), pH 7,5. The column is washed with 10 mM $K_2HPO_4(aq)$, 1,5 M KCl(aq), pH 7,5. The main fraction (20-25 liters, pH 7,2-8,0, cond. 10-15 mS/cm) from each of the step 2 is pooled and stored in a coldroom overnight.

Chromatography sequence

No.	Action	Buffer	Col. vol.
1.	Eqvibration	10 mM K ₂ HPO ₄ (aq), pH 7,5	1
2.	Sample load	Sample	Ca. 5
3.	Wash	10 mM K ₂ HPO ₄ (aq), pH 7,5	2
4.	Wash	10 mM K ₂ HPO ₄ (aq), 6 M urea, pH 7,5	2
5.	Wash	10 mM K ₂ HPO ₄ (aq), pH 7,5	2
6.	Elution	10 mM K ₂ HPO ₄ (aq), 100 mM KCl(aq), pH 7,5	3
7.	Wash	10 mM K ₂ HPO ₄ (aq), 1,5 M KCl(aq), pH 7,5	2
8.	Wash	10 mM K ₂ HPO ₄ (aq), pH 7,5	2

5 STEP 3: AFFINITY CHROMATOGRAPHY, CIBACRONE BLUE FF SEPHAROSE

A Pharmacia BPG-300 column is packed with gel to a volume of 12,5 liters (h=180 mm, diam. 300 mm). Flow is 467 mL/min.

- 10 The pooled fractions from the two step 2 runs are diluted with one volume of 10 mM K₂HPO₄(aq), pH 7,5. The column is equilibrated with 10 mM K₂HPO₄(aq), pH 7,5. After loading of sample and washing with 10 mM K₂HPO₄(aq), pH 7,5, the column is washed with 10 mM K₂HPO₄(aq), 300 mM KCl(aq), pH 7,5 followed by elution of product with 10 mM K₂HPO₄(aq), 450 mM KCl(aq), pH 7,5. The column is then washed with 10 mM
- 15 K₂HPO₄(aq), pH 7,5.

The main fraction (10-15 liters, pH 7,0-8,0, cond.ca. 35 mS/cm) is stored in a coldroom overnight.

- 20 A fermentation process using *E. Coli* and a down stream process for production of aseptic extract containing active recombinant human Porphobilinogen Deaminase (rhPBGD) on a commercial scale. The subsequent purification process for the production of an active bulk product .

STUDY OBJECTIVES

The study objectives are stated in the Study Protocol chapter 3.1 and are shown in the text below.

- Development of a cGMP *E. Coli* fermentation process up to 600 L scale and cell extraction process.
- Development and qualification of in-process analytical tests, rhPBGD tests and specifications. Verification of in process analytical tests - The goal was a fermentation yield of at least 100 mg l⁻¹ rhPBGD and with 50% overall yield of the final purification process.
- Delivery of a frozen, aseptic *E.coli* extract containing active rhPBGD from BioGaia to BioInvent in appropriate, approved containers including small scale sample for verification and acceptance criteria.
- The aseptic rhPBGD extract must meet the criteria of the Specification for aseptic extract for purification of recombinant human Porphobilinogen Deaminase (rhPBGD) as below.

Specification for aseptic extract for purification of recombinant human Porphobilinogen Deaminase (rhPBGD).

The analytical tests are performed on filtered extract.

Description: The extract is buffered with 50 mM sodium phosphate, 1.3 mM EDTA, pH 7,4 and filtered through a 0,22 µm filter.

TEST	METHOD NO.	LIMIT
Content:		
RhPBGD activity (Units/ml)	E 001	> 20
RhPBGD specific activity (Units/mg)	E 001/P 001	> 4.0
Other Tests:		
Protein concentration (mg/ml)	P 001	4.5 – 10.0
SDS-PAGE	S 001	equal to
Bacterial counts (Cfu/ml)	Ph.Eur	reference
		< 10
PH	Ph.Eur.	7.0 – 8.0

Materials and Methods

7.1 Materials

5 Media / substrates used in this study are given below.

In the tables 21-26 below the media compositions for the final developed process are given. From start to the experiment PD14 the thiamine concentration in substrate MM5Y Tc was only 1 mg/l, but was thereafter increased to 10 mg/l starting with experiment PD16.

10

1. M9H-Tc (6 mg^l⁻¹), M9H-Amp (100 mg^l⁻¹) and M9H-Chl (25 mg^l⁻¹) agar plates.

The M9H-Tc (6 mg^l⁻¹) agar plates are used in inoculum procedure while the M9H-Amp (100 mg^l⁻¹) and M9H-Chl (25 mg^l⁻¹) were used for controls of the strain identity.

15

Table 21. Composition of the M9H agar plates

Component	Chemical formula	Conc.	Unit
di-Sodium hydrogen phosphate	Na ₂ HPO ₄	6,00	gl ⁻¹
Potassium dihydrogen phosphate	KH ₂ PO ₄	3,00	gl ⁻¹
Sodium chloride	NaCl	0,50	gl ⁻¹
Ammonium chloride	NH ₄ Cl	1,00	gl ⁻¹
Glucose monohydrate	C ₆ H ₁₂ O ₆ x 1H ₂ O	2,00	gl ⁻¹
Thiamine chloride hydrochloride	C ₁₂ H ₁₈ Cl ₂ N ₄ OS xXH ₂ O	1,00	mg ^l ⁻¹
Magnesium sulphate heptahydrate	MgSO ₄ x 7 H ₂ O	25,0	mg ^l ⁻¹
Bacto Agar	—————	15,0	gl ⁻¹
One of the antibiotics below are added			
Chloramphenicol	C ₁₁ H ₁₂ Cl ₂ N ₂ O ₅	25,0	mg ^l ⁻¹
Ampicillin sodium salt	C ₁₆ H ₁₉ N ₃ O ₄ SNa	100	mg ^l ⁻¹
Oxytetracycline hydrochloride	C ₂₂ H ₂₄ N ₂ O ₉ x HCl	6,00	mg ^l ⁻¹

M9H-Tc (6 mg^l⁻¹) shake flask

20 The M9H-Tc (6 mg^l⁻¹) shake flasks were used in the initial batch experiments and in the inoculum preparation for all experiments.

Table 22. Composition of the M9H-Tc shake flasks.

Component	Chemical formula	Conc.	Unit
di-Sodium hydrogen phosphate	Na_2HPO_4	6,00	gl^{-1}
Potassium dihydrogen phosphate	KH_2PO_4	3,00	gl^{-1}
Sodium chloride	NaCl	0,50	gl^{-1}
Ammonium chloride	NH_4Cl	1,00	gl^{-1}
Glucose monohydrate	$\text{C}_6\text{H}_{12}\text{O}_6 \times 1\text{H}_2\text{O}$	10,0	gl^{-1}
Thiamine chloride hydrochloride	$\text{C}_{12}\text{H}_{18}\text{Cl}_2\text{N}_4\text{OS} \times \text{XH}_2\text{O}$	1,00	mg l^{-1}
Magnesium sulphate heptahydrate	$\text{MgSO}_4 \times 7 \text{H}_2\text{O}$	0,25	gl^{-1}
Oxytetracycline hydrochloride	$\text{C}_{22}\text{H}_{24}\text{N}_2\text{O}_9 \times \text{HCl}$	6,00	mg l^{-1}

MM5Y-Tc (6 mg l^{-1}) shake flask and fermenter medium

- 5 The MM5Y-Tc (6 mg l^{-1}) medium was used for all fermentations with strain PBGD-1 and in the inoculum fermentations in the scale up of the process when the strain PBGD-2 was used. Shake flask cultivations of both strain PBGD-1 and PBGD-2 was performed with this substrate.

10 Table 23. MM5Y-Tc (6 mg l^{-1}) shake flask and fermenter medium

Component	Chemical formula	Conc.*	Unit
Ammonium sulfate	$(\text{NH}_4)_2\text{SO}_4$	2,67	gl^{-1}
Potassium dihydrogen phosphate	KH_2PO_4	3,26	gl^{-1}
di-Potassium hydrogensulfate trihydrate	$\text{K}_2\text{HPO}_4 \times 3 \text{H}_2\text{O}$	2,84	gl^{-1}
tri-Sodium citrate dihydrate	$\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \times 2 \text{H}_2\text{O}$	0,60	gl^{-1}
Yeast extract	-----	5,00	gl^{-1}
Glucose monohydrate	$\text{C}_6\text{H}_{12}\text{O}_6 \times 1\text{H}_2\text{O}$	11,0	gl^{-1}
Magnesium sulfate heptahydrate	$\text{MgSO}_4 \times 7 \text{H}_2\text{O}$	0,25	gl^{-1}
Thiaminechloride hydrochloride	$\text{C}_{12}\text{H}_{18}\text{Cl}_2\text{N}_4\text{OS} \times \text{XH}_2\text{O}$	10,0	mg l^{-1}
Boric acid	H_3BO_3	2,50	mg l^{-1}
Cupper(II)sulfate pentahydrate	$\text{CuSO}_4 \times 5\text{H}_2\text{O}$	10,1	mg l^{-1}
Iron(III)chloride hexahydrate	$\text{FeCl}_3 \times 6\text{H}_2\text{O}$	34,1	mg l^{-1}
Manganese(II)sulfate monohydrate	$\text{MnSO}_4 \times 1\text{H}_2\text{O}$	6,40	mg l^{-1}
Zinc sulfate heptahydrate	$\text{ZnSO}_4 \times 7\text{H}_2\text{O}$	5,00	mg l^{-1}
Cobalt(II)chloride hexahydrate	$\text{CoCl}_2 \times 6\text{H}_2\text{O}$	8,90	mg l^{-1}

Calcium chloride dihydrate	$\text{CaCl}_2 \times 2\text{H}_2\text{O}$	13,9	mg l^{-1}
Sodium molybdate dihydrate	$\text{Na}_2\text{MoO}_4 \times 2\text{H}_2\text{O}$	8,90	mg l^{-1}
Hydrochloric acid, fuming, 37%	HCl	66,0	l l^{-1}
Oxytetracycline hydrochloride	$\text{C}_{22}\text{H}_{24}\text{N}_2\text{O}_9 \times \text{HCl}$	6,00	mg l^{-1}

*Concentration after inoculum.

MM20Y Fermenter medium

The MM20Y Fermenter medium was used in all main fermentations with strain PBGD-1, 5 except for batch PD14.

Table 24. MM20Y Fermenter medium

Component	Chemical formula	Conc.*	Unit
Ammonium sulfate	$(\text{NH}_4)_2\text{SO}_4$	2,67	g l^{-1}
Potassium dihydrogen phosphate	KH_2PO_4	3,26	g l^{-1}
di-Potassium hydrogensulfate trihydrate	$\text{K}_2\text{HPO}_4 \times 3 \text{H}_2\text{O}$	2,84	g l^{-1}
tri-Sodium citrate dihydrate	$\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \times 2 \text{H}_2\text{O}$	0,60	g l^{-1}
Yeast extract	-----	20,0	g l^{-1}
Glucose monohydrate	$\text{C}_6\text{H}_{12}\text{O}_6 \times 1\text{H}_2\text{O}$	11,0	g l^{-1}
Magnesium sulfate heptahydrate	$\text{MgSO}_4 \times 7 \text{H}_2\text{O}$	0,25	g l^{-1}
Thiaminechloride hydrochloride	$\text{C}_{12}\text{H}_{18}\text{Cl}_2\text{N}_4\text{OS} \times \text{XH}_2\text{O}$	10,0	mg l^{-1}
Boric acid	H_3BO_3	2,50	mg l^{-1}
Copper(II)sulfate pentahydrate	$\text{CuSO}_4 \times 5\text{H}_2\text{O}$	10,1	mg l^{-1}
Iron(III)chloride hexahydrate	$\text{FeCl}_3 \times 6\text{H}_2\text{O}$	34,1	mg l^{-1}
Manganese(II)sulfate monohydrate	$\text{MnSO}_4 \times 1\text{H}_2\text{O}$	6,40	mg l^{-1}
Zinc sulfate heptahydrate	$\text{ZnSO}_4 \times 7\text{H}_2\text{O}$	5,00	mg l^{-1}
Cobalt(II)chloride hexahydrate	$\text{CoCl}_2 \times 6\text{H}_2\text{O}$	8,90	mg l^{-1}
Calcium chloride dihydrate	$\text{CaCl}_2 \times 2\text{H}_2\text{O}$	13,9	mg l^{-1}
Sodium molybdate dihydrate	$\text{Na}_2\text{MoO}_4 \times 2\text{H}_2\text{O}$	8,90	mg l^{-1}
Hydrochloric acid, fuming, 37%	HCl	66,0	l l^{-1}
Oxytetracycline hydrochloride	$\text{C}_{22}\text{H}_{24}\text{N}_2\text{O}_9 \times \text{HCl}$	6,00	mg l^{-1}

*Concentration after inoculum.

LB / TSA agar plates

LB or TSA agar plates were for determination of colony forming units (CFU) and as a
5 reference in the plasmid stability controls.

Table 25: Composition of LB agar plates

Component	Concentration	Unit
Tryptone	10,0	g l ⁻¹
Yeast extract	5,00	g l ⁻¹
NaCl	10,0	g l ⁻¹
Bacto Agar	15,0	g l ⁻¹

Table 26: Composition of TSA agar plates

Component	Concentration	Unit
Tryptone	15,0	g l ⁻¹
Soytone	5,00	g l ⁻¹
NaCl	5,00	g l ⁻¹
Bacto Agar	15,0	g/l

10

7.2 Methods**6.2.1 Methods used in II. Development of lab scale fermentation process**

Each cultivation/fermentation started with material from a cryo vial of the intermediary cell
bank (ICB) for either the PBGD-1 (PDWS1:1-80)⁽⁷⁾ or the PBGD-2 strain (PDWS2:1-80)⁽⁷⁾

15 stored at < -70 °C in an ultra freezer. Material from these cryo vials was transferred to
M9H-Tc (6 mg l⁻¹) agar plates (attachment 2, table 1) with a sterile platinum loop. These
plates were then incubated upside down for 23-33 hours at 30 °C.

1-L baffled shake flasks containing 250 ml M9H-Tc (6 mg l⁻¹) substrate or MM5Y-Tc (6 mg l⁻¹)
20 substrate (attachment 2, tables 2 and 3) were inoculated with colonies from 1- 1 ½ agar
plate each, depending on the experimental design. The transfer was made either directly to
the shake flask with a sterile platinum loop or via a test tube containing 9,9 ml sterile 0,9 %
(w/v) NaCl solution. In the latter case the solution was vortexed before inoculum to dissolve
the cell "pellet" properly.

25

Depending on the experimental design the M9H-Tc (6 mg l^{-1}) shake flasks were incubated for 9-14 hours in an air incubator at 135 rpm and 30°C . The OD_{620} at the end of the incubation in these experiments varied between 0,8-1,8. When the experiments only involved shake flask cultivation in either M9H-Tc (6 mg l^{-1}) or MM5Y-Tc (6 mg l^{-1}) substrate the incubation time varied between 12-55 h and the final OD_{620} varied between 0,3-4,0. Depending on experimental design and the volume needed for the subsequent downstream processing development three different fermenters (2 L, 14 L and 20 L) were used. The fermenters were incubated with 250-500 ml broth from the M9H-Tc (6 mg l^{-1}) shake flasks and this in combination with different substrates and volumes in the fermenter made the OD_{620} after the inoculum vary in the range 0,05-2,0. Since the initial glucose concentration always was 10 g l^{-1} this made the length of the batch phase vary between 8,5-15,2 h. When the initial glucose was consumed (end of batch phase) different glucose feed profiles were investigated to control the fermentation. The general fermentation conditions during the development of the lab scale fermentation are given in table 1 below.

Table 27. General fermentation conditions during the development of a lab scale fermentation process

Parameter	Comments
Temperature	Controlled at 30°C
pH	Controlled at 7,0 by titration with 12,5 % NH_3 and 2M H_2SO_4
pO_2 (dissolved oxygen)	Manually controlled $> 20\%$ by changes in aeration and stirrer speed
CO_2	Manually controlled $< 7\%$ by changes in aeration
Aeration	0,5-1,2 VVM
Stirrer speed	500-1200 rpm
$\text{MgSO}_4 \times 7\text{H}_2\text{O}$ feed	Initiated when glucose feed started, constant feed rate $0,3 \text{ g l}^{-1} \text{ h}^{-1}$ based on initial volume

7.2.2 Methods used for III. Scale up of fermentation process.

7.2.2.1 Simulated large scale fermentations.

In the simulated large scale fermentations an extra 9 L inoculum fermentation was performed before the main fermentation to introduce an extra 5-6 generations in the total process. The inoculum fermentation and the main fermentation were performed in the

same fermenter. The procedures for the inoculum preparation on M9H-Tc (6 mg l⁻¹) agar plates and 1 L shake flasks containing 250 ml M9H-Tc (6 mg l⁻¹) substrate were the same as described above in chapter 6.2.1. The inoculum fermenter containing 9 L MM5Y-Tc (6 mg l⁻¹) substrate (attachment 2, table 3) was inoculated with 500 ml broth from two 5 shake flasks and the initial OD₆₂₀ varied between 0,1-0,2. When the OD₆₂₀ reached 7- 8 (9,5-13,0 h) in the 9 L inoculum fermentation 130-150 ml broth was withdrawn and cooled down to 0-8 °C. The volume of the withdrawn broth was calculated to give an initial OD₆₂₀ of 0,1-0,2 in the main fermenter. The remaining broth in the fermenter was removed and the fermenter was rinsed once with 7 L sterile water before 7 L sterile MM20Y substrate 10 (attachment 2, table 4) was transferred to the fermenter. The fermenter was allowed to reach the right fermentation conditions before the fermenter was inoculated with the cold stored broth. This procedure took approximately 1,5 h. After the inoculum the fermentations were performed according to the recently developed lab scale process.

15 7.2.2.2 850 L Scale up fermentations

Four M9H-Tc (6 mg l⁻¹) agar plates were inoculated with material from one cryo vial of strain PBGD-2 and incubated at 30 °C for 23,2-27,2 h. Two 1 L shake flasks, each containing 250 ml M9H-Tc (6 mg l⁻¹) substrate, were inoculated with growth from 1 ½ agar plate each. The shake flasks were incubated in an air incubator at 30 °C and 135 rpm for 12-13,2 h. 20 Broth from the two shake flasks was pooled together in a sterile bottle with connections suitable for sterile transfer to the fermenter. The 14 L fermenter containing 9 L MM5Y-Tc (6 mg l⁻¹) substrate was then inoculated with the 500 ml broth with an OD₆₂₀ of 1,7. The conditions for the 14 L inoculum fermenter are summarised in table 28 below.

25 Table 28. Inoculum fermentation conditions during 850 L Scale up fermentations

Parameter	Value
Temperature	30 °C
pH	7,0
Aeration	0,5 VVM (4,5 l min ⁻¹)
Initial stirrer speed	800 rpm
pO ₂ (dissolved oxygen tension)	> 20 % by manual control of stirrer speed
Fermentation time	8,5-9,2 h (OD ₆₂₀ 7-10)

After 8,5-9,2 h when the OD_{620} in the 14 L fermenter had reached 7,4 –8,6 the broth was transferred to a sterile plastic container suitable for transfer to the 1500 L fermenter containing 850 L MM20Y substrate. The initial OD_{620} in the 1500 L fermenter was 0,17-0,18. Based on the lab scale development and the simulated large scale fermentations the following fermentation strategy was postulated for the 850 L fermentations.

When the initial 10 $g\ l^{-1}$ glucose was consumed (glucose concentration $< 0,5\ g\ l^{-1}$) a stepwise increasing glucose feed (600 $g\ l^{-1}$) profile (3,0 $l\ h^{-1}$ 0-7 h after feed start, 6,0 $l\ h^{-1}$ 7-14 h after feed start and finally an increase to 9,0 $l\ h^{-1}$ for the remaining fermentation) was started. A constant feed (490 $ml\ h^{-1}$) of a 2M $MgSO_4 \times 7H_2O$ feed was started at the same time as the glucose feed. The fermentation conditions in the 1500 L fermenter are summarised in Table 29.below.

Table 29. Conditions in 1500 L fermenter during 850 L Scale up fermentations

Parameter	Comments
Temperature	Controlled at 30 °C
pH	Controlled at 7,0 by titration with 25 % NH_3 and 2M H_2SO_4
Pressure	0,2 bar overpressure
pO_2 (dissolved oxygen)	Manually controlled > 20 % by changes in aeration and stirrer speed
CO_2	Manually controlled < 7 % by changes in aeration
Aeration	0,5-0,8 VVM (425-775 $l\ min^{-1}$)
Stirrer speed	200-400 rpm
Glucose feed (600 $g\ l^{-1}$)	3,0 $l\ h^{-1}$ 0-7 h after feed start, 6,0 $l\ h^{-1}$ 7-14 h after feed start and finally an increase to 9,0 $l\ h^{-1}$ for the remaining fermentation
$MgSO_4 \times 7 H_2O$ feed	Initiated when glucose feed starts, constant feed rate 0,3 $g\ l^{-1}\ h^{-1}$ based on initial volume
Fermentation time	28-30 h

15 7.2.3 Methods used in IV. Development and Scale-up of downstream process

7.2.3.1 Downstream process

During the lab scale development of a down stream process 1-15 L broth was processed (PD07-PD22). A twenty fold scale up to a process taking care of 300 L broth (PD1501 and PD1502) was done to produce material for toxicological and clinical studies.

5

7.2. 3.2 Cell concentration

In all experiments the broth was concentrated 1,5-6,9 times using a cross flow membrane followed by dia filtration (washing) using a buffer to exchange 90-95% of the substrate. The permeate-flux (i.e. flow-rate through the membrane) was controlled between 7-15 $\text{lm}^{-2} \text{h}^{-1}$ using a permeate pump. The filters used in the experiments were a 0,2 μm Sartocan filter (Sartorius) and a 1000 K Biomax filter, v-screen (Millipore), (table 30).

10

Table 30. Filters tested for cell concentration

Filter used	Batch
0,2 μm Sartocan	PD09, PD11, PD12, PD14, PD16, PD19 and PD21
1000 K Biomax, v-screen	PD22, PD1501 and PD1502

After the concentration the cell concentrate was dia filtered with buffer using the same filter and parameters as used for the concentration. The different buffers tested are given in table 5 below. The membrane filtrations were made at ambient temperature in the laboratory scale and controlled at 15-25 °C in during scale up.

20 Table 31. Different buffers tested for cell-concentration

Buffer	Batch
20 mM Tris, 0,67 mM EDTA, pH 8,2	PD09, PD11 and PD12
50 mM Tris, 1,34 mM EDTA, pH 8,2	PD14
50 mM Tris, 1,34 mM EDTA, pH 7,4	PD16
50 mM sodium-phosphate, 1,34 mM EDTA, pH 7,4	PD21, PD22, PD1501 and PD1502

7.2.3.3 Homogenisation

The concentrated and washed cells were homogenised with a laboratory homogeniser (Niro Soavi Panda (10 lh^{-1})) or a production scale homogeniser (Rannie Type LAB 10-51 VH (100 lh^{-1}), APV). The conditions tested were 600-1000 bar, 1-3 passages at ambient

25

temperature. Between passages the temperature was set at 15-25°C in the production scale. Table 32 below summarises the conditions used for each experiment. The homogenate was analysed for rhPBGD-activity and protein concentration (Procedures E001 and P001).

5

Table 32. Conditions tested for homogenisation

Pressure	No of passages	Batch
600 bar	1	PD09
600 bar	2	PD09
600 bar	3	PD21
800 bar	1	PD09, PD11, PD12, PD14, PD16, PD19
800 bar	3	PD19, PD21, PD22, PD1501, PD1502
1000 bar	3	PD19

7.2.3.4 Cell debris removal by membrane filtration

- 10 Before cell debris removal by membrane filtration the homogenate was diluted 2-3 times with the same buffer used for the cell concentration step.

Filters tested for cell debris removal were 0,2 µm Sartocofilter, 1000 K Biomax filter (v-screen) and 500 K Biomax filter (v-Screen). The homogenate was concentrated 1,5-2,5 times and dia filtrated in order to get 80-99 % theoretical yield of rhPBGD, with buffers

- 15 specified in table 33 below. The theoretical yield is a calculated (calculations not shown) yield based on the assumptions that there are no interactions what so ever between the filter surface and the rhPBGD (i.e. 100 % transmission). Since there in practice are many interactions (e.g. fouling, electrostatic forces) the real yield is off course lower. The theoretical yield is mainly used as a to tool to be able to compare results from different
- 20 filtration runs.

Table 33. Buffers tested for cell debris removal.

Buffer	Batch
20 mM Tris, 0,67 mM EDTA, pH 8,2	PD11 and PD12
50 mM Tris, 1,34 mM EDTA, pH 8,2	PD14
50 mM Tris, 1,34 mM EDTA, pH 7,4	PD16
50 mM Na-phosphate, 1,34 mM EDTA, pH 7,4	PD21, PD22, PD1501 and PD1502

The permeate-flux during membrane filtration was controlled between 7-15 $\text{lm}^{-2} \text{h}^{-1}$ using a permeate pump. To prevent oxidation of the extract in the stirred holding tank a stream of nitrogen was flushed over the permeate surface (only PD1502). The Membrane filtrations
5 were made at ambient temperature in the laboratory scale and controlled between 15-25 °C in production scale. The permeate was assayed for rhPBGD activity, protein concentration and SDS-Page (Procedure: E001, P001 and S001).

7.2.3.5 Cell debris removal by centrifugation

10 During the development of a method for cell debris removal centrifugation was initially used in the laboratory scale in order to produce material for further down stream experiments. The homogenised material was centrifuged at 10 000 x g for 20 min in a Sorvall centrifuge (Beckman). The supernatant was collected, saved and assayed for rhPBGD-activity, protein concentration and SDS-Page (Procedures: E001, P001 and S001).

15

7.2.3.6 Final filtration

Permeate from membrane filtrated homogenate and supernatant from centrifuged homogenate were respectively filtered through a 0,22 μm retentive filter (Sartobran or Durapore) into autoclaved containers. The filters were integrity tested and the extract
20 assayed for CFU, rhPBGD-activity, protein concentration and SDS-Page (procedures: K-M50, E001, P001 and S001).

7.2.3.7 Stability studies, rhPBGD-activity

Membrane filtered extract from different batches PD22 and PD1501 were kept at -20°C
25 for several months. Single use aliquots were routinely taken out of the freezer and the rhPBGD-activity was measured and plotted over time (procedure: E001).

7.3.1 Analytical methods

30 *Preparation of undisrupted cells for analysis, Procedure K-M45*

Cells for analysis were prepared and lysed according to procedure K-M45. The procedure is mainly based on a report made by Pieter Jan Oort, IcoGen ⁽⁴⁾.

The sample (10 ml) was centrifuged for 10 min at 7700 x g. The supernatant was poured of and the pellet re suspended in 5 ml 25 mM sodium-phosphate, 0,9 % NaCl, pH 7,4. The
35 new cell suspension was centrifuged for another 10 min at 7700 x g and the pellet was re

suspended in 50 mM sodium-phosphate, 1,34 mM EDTA, pH 7,4. This cell suspension was then sonicated for 3 x 45 s in a Soniprep 150 Sonicator followed by centrifugation for 10 min at 7700 x g. The supernatant was saved and the volume measured. The pellet was re suspended once more in 50 mM sodium-phosphate, 1,34 mM EDTA, pH 7,4, re sonicated for 3 x 45 s and centrifuged at 7700 x g for 10 min. The volume of the supernatant was measured and pooled with the first supernatant. Finally, the total protein concentration and the rhPBGD activity were analysed according to the procedures P 001 and E 001, respectively. From these analyses the specific rhPBGD activity was calculated.

10 *Porphobilinogen Deaminase, rhPBGD Assay, Procedure E001*

rhPBGD-activity was measured according to procedure E001. The procedure is based on the methods published by Jordan et al (1988, 1997) ⁽¹⁾ and ⁽⁶⁾.

Protein determination, Procedure P001

15 Protein concentrations were determined by procedure P001 using the BCA protein assay kit (Pierce). Bovine serum albumin was used as a reference standard (Pierce Instructions, 1997) ⁽⁵⁾.

SDS-polyacrylamide gel electrophoresis, Procedure S001

rhPBGD was analysed by SDS-PAGE on Novex NuPAGE gels according to procedure
20 S001. Electrophoresis System with NuPAGE 4-12 % gels was used for analysis (Novex, 1997, Laemmli 1970) ⁽²⁾.

7.A.I Process start

The final strain was not fully developed by Icogen Inc. at the initiation of the study (1/2/99).

25 Hence the development was started with the intermediary strain designated PBGD-1; an *E. coli* K12 host strain JM105 with genotype endA thi rpsL sbcB15 hsdR4 Δ (lac-proAB) [F' traD36 proAB lacI^q Δ (lacZ) M15] containing the expression plasmid pExp1-M2-BB ⁽⁸⁾.
When the final strain PBGD-2 was delivered, the process developed so far for the intermediary strain PBGD-1 was implemented on this strain. Both the PBGD-1 and 2
30 strains contain the expression plasmid pExp1-M2-BB encoding rhPBGD and the only difference between the strains is that the hem C gene (encoding endogenous PBGD) has been deleted in the PBGD-2 strain to facilitate the purification of rhPBGD.

Strain genotype and information about agar plate and shake flask cultivation from Icogen formed the basis for the first experiments at BioGaia Fermentation. Articles and
35 discussions with HemeBiotech formed the basis for the analysis methods set up.

7.A II. Development of lab scale fermentation

The overall strategy for the development of the fermentation process was outlined as follows. The use of a minimal medium in the inoculum steps should facilitate the stability of the host, and a minimal medium supplemented with yeast extract and peptone should facilitate growth and production in the main fermentation. To reach high cell densities a concentrated glucose feed was used to control the growth rate in the feed phase. In the expression plasmid pExp1-M2-BB the *rop* gene has been deleted ⁽⁸⁾ which means that the expression of rhPBGD can be temperature regulated. Initially it was decided to start with a fermentation temperature of 30 °C, which means that no temperature induction was used. If the productivity at this temperature was unsatisfactory the temperature could be increased to 37 °C or 42 °C to increase the productivity. Oxytetracycline was chosen as selection pressure, but if possible with regard to plasmid stability, the main fermentation should run without any selection pressure at all.

15

7.A.1 Initial batch experiments

The study was initiated 1/2/99 and the intermediary strain PBGD-1 was delivered 4/2/99. An initial M9H-Tc (6 mg l⁻¹) (attachment 2, table 2) shake flask cultivation (PD03) was performed to study the growth in the M9H-Tc (6 mg l⁻¹) inoculum medium recommended by IcoGen Inc. A fermenter medium designated MM5Y-Tc (6 mg l⁻¹) was designed based on BioGaia Fermentations know how from other recombinant *E.coli* fermentations. This medium was first tested in a shake flask cultivation (PD04) before two 1 L batch fermentations (PD05 and PD06) were performed with two variants of the medium. PD06 was performed in MM5Y-Tc (6 mg l⁻¹) substrate complemented with 2 g l⁻¹ tryptone (table 8) to investigate if tryptone could facilitate growth.

25

In a batch cultivation exponential growth continues for a relatively few generations until nutrients are depleted or toxic products accumulates. Due to this growth begins to slow and thereafter the micro organisms enter the stationary phase, where a steady state cell number is reached.

30

In all these batch experiments the initial glucose concentration was 10 g l⁻¹. The parameters defined below were analysed or calculated, and the results are summarised in table 34 below.

35 Maximum growth rate (μ_{\max}) $dX / dt = \mu \times X$, where X = Dry cell weight

Optical density (OD_{620}) The optical density of a cell suspension was measured by light transmission through it. The absorbance was measured at 620 nm with water as reference

5

Dry cell weight (Dw) Dry cell weight is determined from a known volume of cell suspension that is washed free of extraneous materials, dried in an oven, and then weighed

10 Colony forming units (CFU) The CFU technique involves growth of micro-organisms from a suspension on LB or TSA agar media (attachment 2 table 5 and 6). When a single micro organism divides on an agar medium, it forms a colony of cells, which can be seen by the naked eye

15 Glucose concentration Enzymatic analysis with a YSI 2000 instrument

Table 34. Summary of initial batch experiments with strain PBGD-1

	PD03	PD04	PD05	PD06
Cultivation type	250 ml shake flask	250 ml shake flask	1 L fermentation	1L fermentation
Substrate	M9H-Tc	MM5Y-Tc	MM5Y-Tc	MM5Y-Tc + 2 g l ⁻¹ peptone
μ_{max}	0,3 h ⁻¹	0,3 h ⁻¹	0,4 h ⁻¹	0,4 h ⁻¹
Stationary OD_{620}	0,8	3,3	11	8
Stationary Dw	0,17 g l ⁻¹	1,5 g l ⁻¹	3,9 g l ⁻¹	2,9 g l ⁻¹
Stationary CFU	1*10 ⁹ ml ⁻¹	1*10 ⁹ ml ⁻¹	4*10 ⁹ ml ⁻¹	3*10 ⁹ ml ⁻¹
Residual glucose	Not analysed	6 g l ⁻¹	0 g l ⁻¹	0 g l ⁻¹

In the shake flask cultivations growth stops before glucose is consumed and the growth rates and stationary values for OD_{620} , Dw and CFU are lower than in the controlled fermentations. The reason is the decreasing pH in the broth due to acetate formation during the shake flask cultivation (pH was not controlled). The shake flask cultivation in "rich" fermenter medium MM5Y-Tc allowed growth to higher stationary values compared to shake flask cultivation in M9H-Tc medium. In the fermentations growth stops

because the glucose is depleted, and not due to decreasing pH, since pH was controlled at 7.0. A comparison of fermentations PD05 and PD06 indicates that the addition of 2 g l⁻¹ peptone to the MM5Y-Tc medium did not have any positive effect on the growth rate (attachment 3, fig 1.). Hence it was decided to use the MM5Y-Tc medium without any

5 peptone.

The poor correlation between OD₆₂₀, Dw and CFU in the shake flask experiments can be explained by a combination of the following facts. The Dry weight determinations are difficult to perform at such low cell densities and the viability (CFU) of the cells can vary much since the cultivation conditions are not well controlled. Viability is a parameter that is
10 influenced by many parameters in a complex way, and hence it is very difficult to get a good correlation between OD₆₂₀, Dw and CFU, especially in uncontrolled shake flasks with low cell densities.

Final statement: The strain PBGD-1 has the same growth rate (0,3 h⁻¹) in shake flasks with either M9H-Tc (6 mg l⁻¹) or MM5Y-Tc (6 mg l⁻¹) substrate. The growth rate in MM5Y-Tc (6
15 mg l⁻¹) substrate increases to 0,4 h⁻¹ when the strain grows under controlled conditions in a fermenter. The MM5Y-Tc (6 mg l⁻¹) substrate supplements growth up to at least an OD₆₂₀ and a Dw of approximately 10 and 4 g l⁻¹ respectively.

7.A.2 Fed batch fermentations

20

7.A.2.1 Fed batch fermentations with strain PBGD-1

After the establishment of knowledge about some general strain characteristics during the initial batch experiments, the next step was to proceed to fed batch fermentations to reach higher cell densities and product concentrations. In a fed batch fermentation, a
25 concentrated feed of the limiting substrate is fed into the fermenter at a rate ensuring that the respiration and heat evolution does not exceed the capacity of the fermenter the process is designed for. Hence, until any other substrate component is depleted or toxic by product accumulation limits growth, growth can proceed and the stationary phase is avoided. Eventually the increasing starvation of the limiting substrate (due to the increasing
30 biomass) means the fermentation anyway will reach a stationary phase. However this occurs at much higher cell densities than in a batch phase. In this study the stationary phase was never reached in the fed batch fermentations and we are hence from now referring to final values instead.

The strategy was to start a concentrated glucose feed (600 g l^{-1}) when the initial 10 g l^{-1} glucose was reduced below $0,5 \text{ g l}^{-1}$. Three feed batch fermentations were performed to establish a glucose feed profile matching the oxygen transfer in BioGaia Fermentations 1500 L production fermenter and not giving glucose accumulation in the broth. Glucose accumulation was defined as a glucose concentration $> 0,1 \text{ g l}^{-1}$ glucose since this was the lower detection limit for real time glucose measurements in broth. Glucose accumulation occurs when the growth rate varies due to that various compounds in the yeast extract are utilised by the cells. A temporary drop in the growth rate leads to an accumulation of glucose if the glucose feed rate is based on a previous higher growth rate. When glucose accumulates in the broth, acetate production is likely to occur due to overflow metabolism. This means lower yields, but more importantly this can cause growth inhibition as a result of the acetate accumulation. Hence the feed profile has to be adjusted according to the actual growth profile of the strain. The three feed batch fermentations performed with strain PBGD-1 are summarised in table 35 below and graphs with growth and production of rhPBGD versus fermentation time are given in fig 26. and fig 27.

Table 35. Summary of feed batch experiments with strain PBGD-1

	PD09	PD11	PD12
type	7 L fermentation	7 L fermentation	7 L fermentation
Substrate	MM5Y-Tc	MM5Y-Tc	MM5Y-Tc
Batch phase	0-15,2 h	0-13,9 h	0-13,1 h
Feed phase	15,2-20,7 h	13,9-27,5 h	13,1- 31,0 h
Achieved glucose feed profile	70 ml h^{-1} 15,2-20,7 h	78 ml h^{-1} 13,9-15,1 h 0 ml h^{-1} 15,1-16,1 h 43 ml h^{-1} 16,1-17,3 h 53 ml h^{-1} 17,3-18,7 h 72 ml h^{-1} 18,7-27,5 h	43 ml h^{-1} 13,1-17,5 h 90 ml h^{-1} 17,5-18,2 h 60 ml h^{-1} 18,2-19,2 h 0 ml h^{-1} 19,2-22,3 h 75 ml h^{-1} 22,3-23,3 h 28 ml h^{-1} 22,3-31,0 h
Total glucose added	313 g	586 g	430 g
Glucose accumulation	No measurements made during feed phase.	Peak value $3,4 \text{ g l}^{-1}$ between 13,9-16,0 h	Peak values $1,0 \text{ g l}^{-1}$ and $1,9 \text{ g l}^{-1}$ between 13,1- 22,0 h and 22,3-25,0 respectively

Final Dw	14,0 g l ⁻¹	28,8 g l ⁻¹	19,2 g l ⁻¹
Final OD ₆₂₀	37	82	59
Final CFU	Not analysed	3,5*10 ⁹ ml ⁻¹	6*10 ⁹ ml ⁻¹
Final rhPBGD	Not analysed	7,7 Uml ⁻¹	15,3 Uml ⁻¹
Final specific rhPBGD	Not analysed	2,6 Umg ⁻¹	1,8 Umg ⁻¹
Final plasmid stability	Not analysed	LB-Tc: 100% of growth on LB	LB-Tc: 100% of growth on LB

The objective with fermentation PD09 was to use a constant glucose feed (600 g l⁻¹) rate of 75 mlh⁻¹ to reach a high cell density and a good production of rhPBGD. Unfortunately, practical limitations made it impossible to make any measurements during the batch phase.

- 5 However, respiration (data not shown) during the fed batch phase seemed to be low in comparison to the glucose feed rate, and hence there was a possibility that glucose accumulation could have occurred.

- The strategy was to use the same constant glucose feed rate (75 mlh⁻¹) in fermentation
- 10 PD11 and monitor the glucose concentration every hour to ensure that no glucose accumulation occurred. Glucose analyses revealed glucose accumulation (3,4 g l⁻¹) directly after feed start (13,9 h) and hence the glucose feed was stopped until the cells consumed the excess glucose. When the glucose concentration decreased below 0,1 g l⁻¹ (16,1 h) the glucose feed was started again, however at a lower feed rate (43 mlh⁻¹). The glucose feed
- 15 rate was then stepwise increased without any glucose accumulation until a final feed rate of 72 mlh⁻¹ was reached.

- Based on the experience from PD11 it was decided to start the glucose feed (600 g l⁻¹) with a lower constant feed rate (40 mlh⁻¹) to avoid glucose accumulation directly after feed start.
- 20 After 4 h the glucose feed rate should be increased to 75 mlh⁻¹. Despite the lower initial glucose feed rate a slow glucose accumulation occurred at the start of the glucose feed (13,1 h). Due to an operator mistake the feed rate was not decreased and was even increased according to the plan after 17,5 h. As soon as the operator realised the mistake the glucose feed rate was decreased (18,2 h) and stopped after 19,2 h. After 23,3 h the
- 25 glucose feed was started at 75 ml/h but had to be decreased to 28 mlh⁻¹ 1 h later to avoid glucose accumulation. The glucose feed was then kept at 28 mlh⁻¹ for the remaining fermentation.

From Table 35 it can be seen that the different glucose feed profiles determines the outcome of the fermentations. As expected the final OD₆₂₀ and Dw values correlates well to the total amount of glucose added to the fermenter. Again it is seen that the number of viable cells (CFU) is influenced by fermentation conditions in a complex way. The number of viable cells is lower in PD11 than in PD12 even though both OD₆₂₀ and Dw values are approximately 50 % higher in PD11. Also the difference between the rhPBGD expression in PD11 and PD12 is hard to explain, but also this indicates that the production of rhPBGD not only is linked to the cell density but also depends on the physiological status of the cells.

10

Final statement: The fermenter medium MM5Y-Tc (6 mg l⁻¹) supplemented growth up to OD₆₂₀ and Dw values of approximately 80 and 30 g l⁻¹ respectively. The expression levels of the rhPBGD had already at this point in the fermentation development reached acceptable levels. After the initial batch phase it was necessary to find an initial feed rate of the glucose feed (600 g l⁻¹) profile lower than 40 ml h⁻¹ and not giving glucose accumulation.

15

7.A.2.2 Fed batch fermentations with strain PBGD-2

The final strain PBGD-2 had the same expression plasmid pExp1-M2-BB encoding rhPBGD as strain PBGD-1, but the host cell was deleted for the hemC gene to facilitate rhPBGD purification. Since report existed of any differences in the properties of strain PBGD-2 in comparison to strain PBGD-1, the strategy was to implement this strain in the process developed for strain PBGD-1 so far. One difference was that a new inoculum procedure was introduced to shorten the initial batch phase. The main fermenter was inoculated with 500 ml broth from two 250 ml shake flasks cultivated 12 h instead of 250 ml from one incubated for 10 h. Three fed batch fermentations were performed to adjust the fermentation substrate and to adjust the glucose feed profile to the new strain. The results from these fermentations are summarised in table 36 and graphs with growth and production of rhPBGD versus fermentation time are given fig 28. and fig 29.

25

30 Table 36. Summary of feed batch experiments with strain PBGD-2

	PD14	PD16	PD19
Cultivation type	7 L fermentation	7 L fermentation	7 L fermentation
Substrate	MM5Y-Tc + extra yeast extract and	MM20Y	MM20Y

	thiamine		
Initial glucose conc.	10 g l ⁻¹	10 g l ⁻¹	10 g l ⁻¹
Batch phase	0-14,3 h	0-10,8 h	0-10,3 h
Feed phase	14,3-30,0 h	10,8-30,0 h	10,3- 30,0 h
Achieved glucose feed profile	10 mlh ⁻¹ 14,3-15,3 h 20 mlh ⁻¹ 15,3-15,5 h 40 mlh ⁻¹ 15,5-22,3 h 75 mlh ⁻¹ 22,3-30,0 h	25 mlh ⁻¹ 10,8-15,0 h 50 mlh ⁻¹ 15,0-16,8 h 37 mlh ⁻¹ 16,8-17,3 h 0 mlh ⁻¹ 17,3-18,3 h 50 mlh ⁻¹ 18,3-18,7 h 34 mlh ⁻¹ 18,7-19,6 h 37 mlh ⁻¹ 19,6-20,0 h 40 mlh ⁻¹ 20,0-22,6 h 50 mlh ⁻¹ 22,6-23,0 h 55 mlh ⁻¹ 23,0-24,3 h 60 mlh ⁻¹ 24,3-25,3 h 75 mlh ⁻¹ 25,3-30,0 h	23 mlh ⁻¹ 10,3-17,3 h 49 mlh ⁻¹ 17,3-24,3 h 73 mlh ⁻¹ 24,3-30,0 h
Total glucose added	577 g	610 g	652 g
Glucose acc.	No accumulation	Peak values 1,8 g l ⁻¹ and 0,3 g l ⁻¹ between 15,0-18,0 h and 18,7-19,6 h respectively	No accumulation
Final Dw	32,0 g l ⁻¹	Measurement error	Measurement error
Final OD ₆₂₀	87	93	96
Final CFU	9*10 ⁹ ml ⁻¹	30*10 ⁹ ml ⁻¹	37*10 ⁹ ml ⁻¹
Final rhPBGD	39 Uml ⁻¹	42 Uml ⁻¹	66 Uml ⁻¹
Final spec. rhPBGD	3,1 Umg ⁻¹	1,9 Umg ⁻¹	4,8 Umg ⁻¹
Final	Not analysed	102 % of growth on LB	100 % of growth on

plasmid stability			LB
----------------------	--	--	----

- Based on the results from PD12, it was decided to implement a step wise increasing glucose feed profile (25 mlh⁻¹ 0-4 h after feed start, 50 mlh⁻¹ 4-8 h after feed start and finally 75 mlh⁻¹ for the remaining fermentation) to avoid glucose accumulation in fermentation
- 5 PD14. However, during the end of the batch phase a dramatic decrease in the growth rate (observed by OD₆₂₀, pO₂ (dissolved oxygen tension), CO₂ and O₂ trends) was observed (data not shown). Since glucose analysis showed that there was no glucose limitation (glucose conc. was 5,7-0,7 gl⁻¹) the hypothesis was that the initial yeast extract or thiamine was depleted. After 14,5 h an addition of 35 g yeast extract and 21 mg thiamine was made
- 10 and growth was re-established. Hence this test verified the postulated hypothesis. After 16 h another decrease in growth was observed and a second addition of 35 g yeast extract and 21 mg thiamine was made (20,3 h). Growth was again re established and continued until 24 h, when again a weak tendency of decreasing growth was observed. Since interpretation of the growth patterns was difficult at this stage, only 4 g of yeast extract was
- 15 added to study the response of the addition. Since there was no clear response, it was not thought that any more additions were necessary during the rest of the fermentation. The depletion of yeast extract and/or thiamine made it necessary to alter the intended glucose feed profile to avoid glucose accumulation.
- 20 Based on the results from fermentation PD14 a new fermentation substrate MM20Y-Tc (attachment 2, table 4) was designed to avoid depletion of yeast extract or thiamine in fermentation PD16. In the new substrate the concentrations of yeast extract and thiamine was increased from 5 gl⁻¹ and 1 mgl⁻¹ to 20 gl⁻¹ and 10 mgl⁻¹ respectively.
- Plasmid stability and rhPBGD production without oxytetracycline as selection pressure in
- 25 the main fermenter was also tested in PD16. The concentration of oxytetracycline (6 mgl⁻¹) in the inoculum shake flasks was however kept the same as in the earlier experiments.
- The change to the new MM20Y substrate resulted in a smooth growth pattern during the whole fermentation. The intention was to keep the same glucose feed profile as originally planned for fermentation PD14. Hence the glucose feed was started at 25 mlh⁻¹ after 10,8
- 30 h. After 15 h when the glucose feed rate was increased to 50 mlh⁻¹ glucose started to accumulate in the broth. The glucose feed rate was decreased after 16 h (0,7 gl/l) and stopped after 17 h since the glucose concentration in the broth still was increasing (1,8 gl⁻¹). After 18 h when the accumulated glucose was consumed (< 0,1 gl⁻¹) the glucose feed

was started at 50 mlh^{-1} and again glucose accumulation was observed ($0,3 \text{ gl}^{-1}$). The feed rate was now decreased to and kept at 34 mlh^{-1} until the excess glucose was consumed and a stable situation without glucose accumulation was established. Finally the glucose feed rate was increased step wise to a final value of 75 mlh^{-1} according to table 36 above.

5

To avoid the glucose accumulation observed in PD16 the glucose feed profile was changed for PD19 to the following; 25 mlh^{-1} 0-7 h after feed start, 50 mlh^{-1} 7-14 h after feed start and 75 mlh^{-1} during the rest of the fermentation. Since there was no sign of plasmid loss or decrease in rhPBGD production in PD16 it was decided to exclude oxytetracycline in the main fermenter also in experiment PD19.

In PD19 the new glucose feed profile did not give raise to any glucose accumulation, and hence no changes in the planned glucose profile was necessary. As in PD16 no plasmid loss was observed and the rhPBGD production reached even higher values than in PD16. Also in these fermentations the final OD_{620} values correlates well with the total amount of glucose added to the fermentation. A measurement error makes it impossible to compare the results of the dry weight determinations. The number of viable cells (CFU) was greatly improved when changing to the new substrate composition MM20Y, and shows a fairly good reproducibility between PD16 and PD19. There is no clear trend for the rhPBGD production, but it seems to be a tendency that the rhPBGD production is positively influenced by both the increases in cell density and viability in fermentations PD16 and PD19.

Final statement: The fermenter medium MM5Y-Tc (6 mg l^{-1}) was not able to supplement strain PBGD-2 up to the OD_{620} and Dw values reached with strain PBGD-1. The new designed fermenter medium MM20Y with increased concentrations of yeast extract and thiamine was able to supplement growth up to OD_{620} and Dw of approximately 90 and 30 gl^{-1} respectively. The expression of rhPBGD was in these fermentations at very good levels. After the initial batch phase the final glucose feed (600 gl^{-1}) profile for a 7 L fermentation was designed like follows: 25 mlh^{-1} 0-7 h after feed start, 50 mlh^{-1} 7-14 h after feed start and 75 mlh^{-1} 14-21 h after feed start. There were no negative effects on plasmid stability and rhPBGD expression when excluding oxytetracycline as selection pressure in the main fermenter.

7.A.3 III. Scale up of fermentation

The scale up part of the development was divided into two parts. First simulated large scale fermentations were performed in laboratory fermenters to study the effect of the increasing number of generations on plasmid stability and product quality. These tests were followed by the actual 850 L fermentations to investigate the effects from increasing the fermentation scale on plasmid stability and product quality.

BioGaia Fermentations 1500 L production fermenters are normally inoculated with broth from one 14 L fermenter. To mimic the fermentations PD14-PD19 the OD₆₂₀ after inoculation should be approximately 0,1. When using a working volume of 9 L in the 14 L fermenter the final OD₆₂₀ necessary to achieve the same inoculum conditions in 850 L can be calculated as follows

$$15 \quad OD_{620} \times 9 \text{ L} = 0,1 \times 850 \text{ L} \quad \rightarrow \quad OD_{620} = 9,4$$

Inoculating the 14 L fermenter containing 9 L substrate with 500 ml broth (two 1 L shake flasks) with an OD₆₂₀ of approximately 1,0 gives an OD₆₂₀ initial of approximately 0,1-0,2. With a growth rate of 0,4 h⁻¹ it will take 9-11 h to reach an OD₆₂₀ of about 9. Since a growth rate of 0,4 h⁻¹ is equivalent to a generation time of 1,7 h (ln2/0,4) this corresponds to 5-6 extra generations compared to the earlier lab scale fermentations.

An estimation of the number of generations in different steps of the entire process is given in table 11 below. The extra inoculum fermentation increases the total number of generations in the process with 15-25 %, an increase that could have effects on plasmid stability and product quality.

Table 37. Estimated number of generations in different process steps

Process step	Estimated number of generations
M9H-Tc agar plates	8 - 12
0,25 L M9H-Tc Shake flasks	4 - 5
9 L Inoculum fermentation	5 - 6
850 L Main fermentation	9 - 11
Σ Total process	26 - 34

7.A.3.1 Results from simulated large scale fermentations

It was decided to use a rich substrate MM5Y-Tc (6 mg l^{-1}) in the inoculum fermenter for two reasons. The first reason was that an increase in the growth rate from $0,3 \text{ h}^{-1}$ to $0,4 \text{ h}^{-1}$

- 5 would decrease the process time with approximately 5 h. The second reason was the hesitation whether or not the M9H-Tc (6 mg l^{-1}) substrate could support growth up to OD_{620} values in the range 7-10. Table 38 below compares the results from the simulated large scale fermentations with the developed lab scale process and the growth and rhPBGD production versus time are shown in, fig 30 and fig 31.

10

Table 38. Results from simulated large scale fermentations in comparison to the developed lab scale process.

	PD19	PD21	PD22
Cultivation type	7 L fermentation	Simulated large scale: 9 L inoculum + 7 main fermentation	Simulated large scale: 9 L inoculum + 13 L main fermentation
Substrate	MM20Y	MM20Y	MM20Y
Initial glucose conc.	10 g l^{-1}	10 g l^{-1}	10 g l^{-1}
Batch phase	0-10,3 h	0-9,25 h	0-8,50 h
Feed phase	10,3- 30,0 h	9,25-30,0 h	8,50-30,0 h
Achieved glucose feed profile	23 ml h^{-1} 10,3-17,3 h 49 ml h^{-1} 17,3-24,3 h 73 ml h^{-1} 24,3-30,0 h	20 ml h^{-1} 9,25-11,0 h 24 ml h^{-1} 11,0-13,3 h 25 ml h^{-1} 13,3-16,3 h 41 ml h^{-1} 16,3-20,3 h 43 ml h^{-1} 20,3-23,3 h 65 ml h^{-1} 23,3-25,0 h 70 ml h^{-1} 25,0-30,0 h	43 ml h^{-1} 8,5-10,0 h 40 ml h^{-1} 10,0-13,0 h 50 ml h^{-1} 13,0-14,0 h 45 ml h^{-1} 14,0-15,3 h 60 ml h^{-1} 15,3-16,0 h 80 ml h^{-1} 16,0-17,0 h 85 ml h^{-1} 17,0-22,3 h 150 ml h^{-1} 22,3-23,3 h 121 ml h^{-1} 23,3-24,0 h 135 ml h^{-1} 24,0-25,0 h

			130 mlh ⁻¹ 25,0-26,0 h 135 mlh ⁻¹ 26,0-27,0 h 150 mlh ⁻¹ 27,0-28,0 h 130 mlh ⁻¹ 28,0-29,0 h 150 mlh ⁻¹ 29,0-30,0 h
Total glucose added	652 g	622 g	1303 g (702 g)*
Glucose acc.	No accumulation	No accumulation	No accumulation
Final Dw	Measurement error	36,9 gl ⁻¹	38,8 gl ⁻¹
Final OD ₆₂₀	96	96	95
Final CFU	37*10 ⁹ ml ⁻¹	21*10 ⁹ ml ⁻¹	46*10 ⁹ ml ⁻¹

Table 38. Results from simulated large scale fermentations in comparison to the developed lab scale process (continued).

Final rhPBGD	66 Uml ⁻¹	66 Uml ⁻¹	69 Uml ⁻¹
Final spec. rhPBGD	4,8 Umg ⁻¹	4,2 U mg ⁻¹	3,8 U mg ⁻¹
Final plasmid stability	100 % of growth on LB	100 % of growth on LB	96 % of growth on LB

*recalculated to a 7 L fermentation

5

There was still a rather good correlation between the amount of glucose added to the fermentation (when adjusted for the differences in volume) and the final Dw and OD₆₂₀ values. When comparing the results from fermentations PD21 and PD22 with PD19 there is a very good reproducibility in all measured parameters. Since PD19 when the final glucose feed profile was fixed the reproducibility in CFU values has improved greatly. This is due to that we now have more controlled and similar conditions during the fermentations. The levels of rhPBGD expression and the plasmid stability are very good. Based on the SDS Page assay it was not possible to detect any differences in the produced rhPBGD from PD21 and PD22 when compared to PD19.

15

Final statement: The 5-6 extra generations introduced in the inoculum procedure do not have any influence on the growth pattern, productivity or quality of the produced rhPBGD. The fermentation process seems to be very reproducible.

5 7.A.3.2 850 L Scale Up fermentations

Two scale up fermentations were performed according to the outline of the simulated large scale fermentations. The only differences were that all 9 L broth from the inoculum fermenter were used for inoculation and that the broth was used directly for inoculation instead of being stored at 0-8 °C for approximately 1,5 h as in the simulated large scale fermentations. Results from the scale up fermentations are summarised in table 39 and the growth and rhPBGD production versus time are shown in fig32 and fig 32.

Table 39. Results from 850 L Scale up fermentations in comparison to the developed lab scale fermentation

	PD19	PD1501	PD1502
Cultivation type	7 L fermentation	Scale Up fermentation 9 L inoculum + 850 L main fermentation	Scale Up fermentation 9 L inoculum + 850 L main fermentation
Substrate	MM20Y	MM20Y	MM20Y
Initial glucose conc.	10 g l ⁻¹	10 g l ⁻¹	10 g l ⁻¹
Batch phase	0-10,3 h	0-9,0 h	0-8,5 h
Feed phase	10,3- 30,0 h	9,0-30,0 h	8,5-28,0 h
Glucose feed profile	23 mlh ⁻¹ 10,3-17,3 h 49 mlh ⁻¹ 17,3-24,3 h 73 mlh ⁻¹ 24,3-30,0 h	Averaged values from process data 3,3 lh ⁻¹ 9,00 - 16,5 h 5,5 lh ⁻¹ 16,5 - 23,5 h 10,5 lh ⁻¹ 23,5 - 30,0 h	Averaged values from process data 3,3 lh ⁻¹ 9,00 - 15,5 h 5,7 lh ⁻¹ 15,5 - 22,5 h 9,8 lh ⁻¹ 22,5 - 28,0 h
Tot.	652 g	89,5 kg (737 g)*	79,9 kg (658 g)*

glucose added			
Glucose acc.	No accumulation	No accumulation	No accumulation
Final Dw	Measurement error	40,7 gl ⁻¹	37,7 gl ⁻¹
Final OD ₆₂₀	96	111	106
Final CFU	37*10 ⁹ ml ⁻¹	89*10 ⁹ ml ⁻¹	140*10 ⁹ ml ⁻¹
Final rhPBGD	66 Uml ⁻¹	62 Uml ⁻¹	59 Uml ⁻¹
Final spec. rhPBGD	4,8 Umg ⁻¹	3,8 U mg ⁻¹	4,1 U mg ⁻¹
Final plasmid stability	100 % of growth on LB	100 % of growth on LB	99 % of growth on LB

*Recalculated to 7 L

- To get conditions similar to batch PD1501 in the down stream processing it was decided to stop the fermentation at an OD₆₂₀ close to the final value in fermentation PD1501. Hence
- 5 PD1502 was stopped after 28 h instead of 30 h. One common explanation to this kind of differences in growth pattern are quality differences between different batches of yeast extract, even from the same manufacturer.
- When comparing the fermentations PD1501 and PD1502 with PD19 there is a very good reproducibility in all measured parameters, except for the CFU values. It seems like the
- 10 amount of viable cells increases dramatically when increasing the fermentation volume (i.e. changing from homogeneous well mixed conditions in a lab scale fermenter to a inhomogeneous production fermenter where the cells encounter constantly fluctuating conditions). This is a phenomenon that has been reported for other fermentation processes as well, but on the other hand there are also examples of the opposite. The viability of a
- 15 population of cells are influenced by a many parameters in a complex way and is impossible to explain the variations based on these very limited experiments performed during this study. No changes in the pattern on the SDS Page gel indicated any changes in the quality of the produced rhPBGD. A comparison of the SDS-Page gels from PD22 (13 L), PD1501(850 L) and PF1502 (850 L) are shown in fig 34. Also the plasmid stability and
- 20 the rhPBGD productivity was unaffected by the increase in scale.

Final statement: The scale up from a 7-13 L lab fermentation to a 850 L production fermentation do not have any influence on the growth pattern, productivity or quality of the produced rhPBGD. The developed process is very reproducible even when comparing lab scale and large scale fermentations.

5

7.A.4 Results from IV. Development and Scale-up of down stream process

7.A.4.1 Cell concentration

In a series of experiments the broth was concentrated 1,9-6,9 times. The lower
10 concentration factors reflected problems with clogging of the membrane surface. The optimal concentration factor was about 2. Such a low concentration factor demands a large buffer volume for dia filtration. The dia filtration volume has to be at least two times the concentration volume to exchange about 90 % of the substrate in the broth. No differences were seen in dia filtration results using different buffers (table 31).

15

Final statement: As a 1000 K filter was chosen for the cell debris removal (see 7.4.3) the same filter was used for cell concentration for practical reasons when scaling up to production scale. The broth was then concentrated around 2 times with a dia filtration volume of two times the concentration volume with a buffer consisting of 50 mM sodium-
20 phosphate, 1,34 mM EDTA, pH 7,4. Permeate-flux was set at $15 \text{ l m}^{-2}\text{h}^{-1}$ and the temperature controlled between 15 and 25°C (PD1501-PD1502).

7.A.4.2 Homogenisation

25 There were no significant differences in protein concentration and rhPBGD-activity using different pressures and number of passages. However, after three passages at 800 bar, disruption of cells was considered to be optimum based on viscosity and results from cell debris removal. After one passage the homogenate was highly viscous due to the presence of unfragmented genomic DNA. The viscosity decreases significantly after further
30 passages from shearing of the DNA into smaller parts. This is important to prevent clogging on the membrane surface. An Increasing number of passages gave less viscous homogenate (table 40).

Final statement: Parameters chosen for scaling up to production scale were 800 bar, 3
35 passages since disruption of cells was then maximised. Homogenate between passages

was kept in a tank with a refrigerated jacket. To facilitate the cell debris removal, it was decided to dilute the homogenate 2,5 times after the third passage. A buffer consisting of 50 mM sodium-phosphate, 1,34 mM EDTA, pH 7,4 was used for the dilution. Between passages temperature at the homogenate in the tank was controlled between 15 and 25°C

5 (PD1501-PD1502).

Table 40. Homogenisation of E-coli cells containing rhPBGD-activity, Batch PD19

Pressure Bar	Number of passage s	Total protein mg/ml	Activity U/ml	Comments
800	1	14,2	67	Viscous
600	3	14,6	72	Not viscous
800	3	15,5	79	Not viscous
1000	3	14,8	76	Not viscous

7.A.4.3 Cell debris removal

- 10 When centrifuged extracts exceeded 10 mg protein ml⁻¹ problems with precipitation and a significant decrease in pH occurred. Membrane filtered extracts did not give the same problems with precipitation as centrifuged extracts. When testing different buffers with different pH during cell debris removal it was obvious that the ionic strength of the buffer had to be at least 50 mM, with a pH around 7,4 to avoid later decrease in pH. It was also
- 15 concluded that rhPBGD was more stable in buffers with a pH around 7,4 than pH 8,2. The final choice of buffer was 50 mM sodium-phosphate, 1,34 mM EDTA, pH 7,4.

The produced extract was dark pink in colour. When standing at room temperature with vigorous stirring extract became brownish-red in a couple of hours. This was probably due

20 to an oxidation process taking part in the extract.

The transmission of proteins through the membrane was low using a 0,2 µm filter. The rhPBGD activity yield was only around 20 %. The yield was however affected positively by changing the homogenisation parameters from one passage to three passages (table 41).

25

Table 41 . Summary of rhPBGD yield after cell debris removal using different pressures during homogenisation. Filter used was 0,2 µm, Batch PD19.

Pressure Bar	Number of passages	Yield Total protein mg/ml	Yield Activity U/ml	Specific activity U/mg protein
800	1	1,2	4	3,3
600	3	1,6	7	4,3
800	3	3,5	16	4,6
1000	3	1,7	10	5,9

Exchanging the micro filter (0,2 μm) for ultra filters (500 K and 1000 K) resulted in less fouling at the filter surface and protein concentration increased to acceptable yields (Table 16). The smaller membrane area and flatter surface minimised product hold up and adsorption, which in this case increases yields. Furthermore, the transmission of total protein seems to be lower than the transmission of rhPBGD when using an ultra filter (1000 K), resulting in a higher specific activity (table 16). In all experiments the homogenate was dia filtered to the same theoretical yield (90%) to enable comparison of the results.

- 10 Final statement: For scaling up to production scale a 1000 K filter was chosen for cell debris removal since this filter gave a good yield of rhPBGD with a high specific activity. Diluted homogenate was decided to be concentrated approximately 2,5 times and then dia filtered with 50 mM sodium-phosphate, 1,34 mM EDTA, pH 7,4 to get a theoretical yield of rhPBGD of about 95 %. Nitrogen was flushed over the permeate surface in order to prevent oxidation (PD1502). Permeate-flux was set at $15 \text{ l m}^{-2}\text{h}^{-1}$ and the temperature was set at 15- 25°C (PD1501-PD1502).

Table 42. Summary of rhPBGD yield from Batch PD21 using different filters during cell debris removal. Homogenisation parameters: 800 bar, 3 passages. The yield of rhPBGD from membrane filtration was compared to centrifuged material.

Cell debris removal by	Yield		Yield		Specific activity U/mg protein
	Total protein mg/ml	%*	Activity U/ml	%*	
0,2 μm filter	2,2	30	7	21	3,3
500 K filter	4,8	65	22	69	4,6
1000 K filter	3,1	42	21	66	6,8
centrifuged	—	80	—	90	5,1

* Yield is given in % compared to homogenate.

7.A.4.4 Final filtration

Membrane filtered extracts contained less particles and was thereby easier to filter than centrifuged extract, where problems with clogging on the filter surface occurred. The clogging made it difficult to perform integrity tests. A white slippery precipitate was always seen in extract before the final filtration. When analysing the dissolved precipitate spectrophotometrically at OD_{260} / OD_{280} resulting in a ratio near 2, it was concluded that it contained nucleic acid.

10 7.A.4.5 Scale up of down stream process (PD22, PD1501 and PD1502)

The entire final process was at first tested at a 15 L scale (batch PD22) ending with a rhPBGD yield of 75 %. When scaling up to 300 L (PD1501) problems with precipitation in the broth occurred and the rhPBGD yield decreased to 46 %. When processing batch PD1502 no precipitation was seen and the rhPBGD yield increased to 77 %. The results are summarised in tables 43 and 44.

The low yields of rhPBGD from batch PD1501 was probably due to several factors: Using the same filter unit at cell concentration and cell debris removal commonly saves both money and time but when a white precipitate occurred in the broth it resulted in problems cleaning the filter between cell concentration and cell debris removal. The composition of the white insoluble precipitate was analysed and the results are shown in table 19. To avoid the precipitate formation in PD1502 the substrate preparation was carefully monitored. No new component was added until the former component was completely dissolved. No precipitate was formed in batch PD1502.

The filter area was small in comparison to the processed volume, which increased the chances for clogging on the filter surface. Dia-filtration was only performed to achieve a theoretical yield, rhPBGD, of 90 %.

In batch PD1502 the concentration of rhPBGD in the extract was low compared to batch PD22 but the yield was slightly higher. The lower concentration was due to an operator mistake using an increased dia filtration volume at cell debris removal during the process of PD1502. If a smaller dia filtration volume during cell debris removal in batch PD1502 had been used it would have resulted in a higher concentration of rhPBGD but the yield had then decreased.

The concentration of rhPBGD was also higher in starting material (homogenate) from batch PD22 and PD1501 than from PD1502. Furthermore the specific activity in the extracts from batch PD22 and PD1501 are higher than in the extract from PD1502.

- 5 To compare rhPBGD-protein amount with rhPBGD-activity, samples from different stages in the process were analysed with SDS-PAGE according to procedure S001. In all batches tested (PD22, PD1501 and PD1502) a major band was seen in a position corresponding to a molecular weight around 40kD and with almost the same mobility as the rhPBGD-His standard. We therefore conclude that this band corresponds to rhPBGD and is responsible
- 10 for the rhPBGD-activity measured in the samples 34.

Table 43. Parameters of the final extraction process for the last laboratory batch PD22 and the two production batches PD1501 and PD1502

Batch	PD22	PD1501	PD1502
Volume broth processed (l)	15	300	300
Filter used for Cell concentration	1000K, 0,5 m ²	1000K, 4 m ²	1000K, 8 m ²
Permeate Flow rate (Flux) Cell concentration	11,0 l m ⁻² h ⁻¹	14,5 l m ⁻² h ⁻¹	13,9 l m ⁻² h ⁻¹
Homogenisation parameters	800 bar, 3 passages	800 bar, 3 passages	800 bar, 3 passages
Filter used for Cell debris removal	1000K, 0,5 m ²	1000K, 4 m ²	1000K, 8 m ²
Permeate Flow rate (Flux) Cell debris removal	11,6 l m ⁻² h ⁻¹	11,5 l m ⁻² h ⁻¹	12,0 l m ⁻² h ⁻¹
CF (concentration factor cell debris removal)	2,0	2,2	2,5
Dia-filtration cell debris removal % theoretical yield	93	90	98
Volume extract after cell debris removal	25 liter	450 liter	670 liter
Dilution factor extract volume /broth volume	1,7	1,5	2,2
Filter used for sterile-filtration	Sartobran P, 0,2 m ²	Durapore CVGL71	Durapore CVGL71

Table 44. Results from the extraction of the last laboratory and the two production batches using the final process.

Batch	PD22	PD1501	PD1502
Homogenate protein (mg/ml)	17,4	18,6	15,0
Homogenate activity (U/ml; kU)	82; 1280	71; 25420	51; 17950
Specific activity, homogenate (U/mg)	4,7	3,8	3,4
Extract protein (mg/ml; g)	7,5; 261	5,3; 2370	5,0; 3300
Extract (U/ml; kU)	38; 950	25; 11620	21; 13860
Yield protein from homogenate (%)	72	36	62
Yield activity from homogenate (%)	74	46	77
Specific activity, extract (U/mg)	5,1	4,7	4,2

5 Table 45. Composition of the white insoluble precipitate from broth (Batch PD1501).

Composition	mg/kg dry weight
Sodium (Na)	510
Potassium (K)	5 800
Calcium (Ca)	900
Magnesium (Mg)	190 000
Phosphate (P)	230 000

Final Statement: The scale up of the extraction process from a 15 L laboratory scale to a 300 L production scale, with a longer product hold up, seems not to have any important influence on the quality of the extract produced. However, it should be mentioned that in 10 stability studies, extract from the production scale batch PD1501 was more stabile over time in the freezer than the laboratory batch PD22 (See 7.A.4.6).

7.A.4.6 Stability studies, rhPBGD-activity

15 When the final extract was stored in freezer (-20°C) extract from the final laboratory batch (PD22) gave a decrease in activity of approximately 10 percent monthly while extract from

a production batch (PD1501) resulted in a 3 percent decrease in activity over the same time (Fig 35).

Evaluation and conclusions

5

We have developed a fermentation and down stream process for production of sterile filtered permeate containing recombinant human Porphobilinogen Deaminase (rhPBGD) on a commercial scale. The process is briefly outline as follows.

- 10 The fermentation process is started with cells from cryo vials stored in an ultra freezer at a temperature $< -70^{\circ}\text{C}$. In all inoculum steps (agar plates, shake flasks and inoculum fermenter) 6 mg l^{-1} oxytetracycline is used as selection pressure to ensure a good plasmid stability. In all process steps the temperature is 30°C . The minimal medium agar plates are inoculated with cells from the cryo vial and are incubated for $24 \pm 4\text{ h}$. Two 1 L shake flasks
- 15 are inoculated with growth from $1\frac{1}{2}$ agar plate each and are incubated for $13 \pm 1\text{ h}$ before the broth is pooled and used to inoculate an 14 L inoculum fermenter. The inoculum fermenter containing 9 L minimal medium supplemented with 5 g l^{-1} yeast extract. When OD_{620} is 7-10 (after approximately 9 h) the broth is transferred to the 1500 L production fermenter containing 850 L minimal medium supplemented with 20 g l^{-1} yeast extract and 10
- 20 mg l^{-1} thiamine. No selection pressure is used in the production fermenter. After an initial batch phase (approximately 8 h) growth is controlled by a stepwise increasing glucose feed (600 g l^{-1}) profile. Totally about 120 L glucose (600 g l^{-1}) solution is fed into the fermenter and together with the NH_3 (25% w/w) and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ also fed into the fermenter this gives a final fermentation volume of about 1000 L. The fermentation is stopped when OD_{620}
- 25 is 100 ± 20 (approximately 30 h) and the broth is cooled down to $20\text{--}25^{\circ}\text{C}$ before downstream processing starts. At the end of fermentation the dry cell weight is about 40 g l^{-1} and the rhPBGD activity is about 60 U ml^{-1} .

The process described for rhPBGD extraction from the PBGD-2 strain of *E.coli* cells is

30 based largely on the use of membrane filtration with a 1000 K ultra filter both for cell concentration and cell debris removal. During cell concentration and cell debris removal the temperature is controlled between 15 and 25°C . Disruption of cells is accomplished by homogenisation at 800 bar for 3 passages at ambient temperature. Between passages homogenate is kept in a tempered ($15\text{--}25^{\circ}\text{C}$) tank. Finally extract is filtered through a $0,22$

35 μm retentive filter into autoclaved containers.

By means of the process developed, rhPBGD can be extracted from 300 L broth in less than 30 hours with an overall yield of about 75 % with a concentration in the extract of approximately 25-30 U/ ml.

- 5 The 850 L fermentation process developed gives a very good and reproducible expression of rhPBGD without temperature induction. Plasmid stability is good even without selection pressure in the 1500 L production fermenter. No negative Scale up effects was encountered during Scale up. The 300 L down stream process is reproducible and has a good overall yield. The down stream process is foreseen to be easy to further scale up to
- 10 give a process taking care of all 1000 L broth produced in the fermentation. Hence the developed process are therefore at this stage found suitable for large scale production of rhBPGD.

List of figures

- 15 Fig 25. Comparison of fermentations PD05 and PD06 with strain PBGD-2.
Fig 26. Comparison of fermentations PD09, PD11 and PD12
Fig 27: Comparison of fermentations PD09, PD11 and PD12 with strain PBGD-1.
Fig 28. Comparison of fermentations PD14, PD16 and PD19 with strain PBGD-2.
Fig 29. Comparison of fermentations PD14, PD16 and PD19 with strain PBGD-2.
- 20 Fig 30. Comparison of fermentations PD19, PD21 and PD22 with strain PBGD-2.
Fig 31. Comparison of fermentations PD19, PD21 and PD22 with strain PBGD-2.
Fig 32. Comparison of fermentations PD19, PD1501 and PD1502.
Fig 33. Comparison of fermentations PD19, PD1501 and PD1502 with strain PBGD-2.
Fig 34. Summary of Fermentation and down stream process visualised by SDS-Page.
- 25 Comparison between different samples
Fig 35. Stability studies: Single use aliquots of extract were routinely taken out of the freezer (-20°C) and the rhPBGD-activity was measured and plotted over time.

Example 8

30

Cloning of rhALAD

The goal of the study is to clone and sequence the cDNA encoding the erythropoietic form of the human enzyme delta-aminolevulinic acid dehydratase (ALAD). This study was

35 undertaken with a long term view to develop an expression system for producing

recombinant human delta-aminolevulinic acid dehydratase (rhALAD) in *Escherichia coli* (*E. coli*).

ALAD is the second enzyme of the heme biosynthetic pathway. Human ALAD is encoded
5 by a single gene localized to the chromosome 9q34 region (1). Although alternative
splicing results in the formation of erythroid-specific and housekeeping transcripts the
differences are restricted to the 5' untranslated sequences and both transcripts encode
identical polypeptides in all cell types (1). The cDNA sequence of human ALAD is
published (2) and the aim of this study is to clone the cDNA for human ALAD from a
10 suitable source and confirm its identity to the published sequence.

Study objectives

Clone cDNA encoding the 330 residue human ALAD polypeptide (2) from a suitable source
by PCR amplification.

15

**Sequence at least three independent clones in both directions to find to a clone that
matches the published sequence.**

A PCR amplification strategy was used to clone the ALAD from human spleen cDNA. The
amplified product obtained by using the primers ICO549 and ICO550 (Figure 36) was
20 digested with *EcoR* I and *Hind* III and cloned into pBluescript II SK- cut with the same
enzymes (Figure 37). Four clones ensuing from the ligation (pBlueAlaD-1-4) were
sequenced.

Materials and Methods

25 8.1 PCR and Cloning

As the same ALAD polypeptide is expressed in all cell types (1) any tissue can serve as a
source for cloning. Spleen cDNA (made by Donald Rao using BRL Superscript II with 500
ng Clontech poly-A RNA from a pool of different donors, catalogue # 6542-1, in 20 l
reaction volumes per manufacturer's instructions) was used. One l of cDNA
30 (approximately 25 g) was amplified with Advantage cDNA polymerase mix (Clontech
Catalogue # 8417-1) with 0.2mM dNTP and 0.4 M each of ICO549 (5'
ATCCATGAATTCCACGCAATGC AGCCCCAGTC 3') and ICO550 (5'
AGTCGTAAGCTTGCCTGGCA CTGTCCTCCATC 3') in 50 l reaction volumes. Two
cycle PCR was used with an initial heat denaturation step at 94°C for 100 seconds followed
35 by 28 cycles of 96°C for 20 seconds and 72°C for 2 minutes. A final extension of 7 minutes

at 72°C was used at the end to ensure that the extension products were filled out. One of this PCR mix was again amplified exactly as described above and cloned into pBluescript II SK- (Stratagene, catalogue # 212206), linearized with *EcoR* I and *Hind* III after purification (using GEANECLEAN III, from BIO 101 catalogue # 1001-600) and digestion with the same two enzymes (see Figure 37, A and B).

8.2 Sequencing

Four plasmid clones from the above ligation viz. pBlueAlaD-1-4 were sequenced with the Big Dye terminator cycle sequencing kit from PE/ABI catalogue # 4303152. Three vector primers, ICO383 (5' GTAATACGACTCACTATA GGGC 3'), ICO384 (5' CTAAAGGGAACAAAAGCTGGAG 3') and IC0618 (5- GCGCGTAATACGACTCACTA 3) and two ALAD-specific primers, ICO616 (5' CCTACGCTGTGTCTTGATCT 3') and ICO617 (5' GGCTT CACCATGAGCATGTC 3') were used. The results are tabulated in Table 46.

Table 46 Summary table of sequencing results

Clone #	Nucleotide change	Amino acid Change
BlueAlaD-1	-	-
BlueAlaD-2	-	-
PBlueAlaD-3	168, T to C 414, C to T 463, T to C 866, C to T	56, Y (silent) 138, N (silent) 155, L (silent) 289, A to V
PBlueAlaD-4	180, T to C	56, y (silent)

Reporting and Results

20

The inserts in all four clones confirmed to be ALAD by sequence analysis. The results are shown in Table 1. As seen, two of the clones completely match the published sequence (2). The other two have changes, most of which are silent. Without a larger sampling volume it is difficult to distinguish between allelic variation and PCR/cloning artifacts. The

ALAD insert from pBlueAlaD-2 was used for expression purposes and its sequence is shown in seq. 14.

Evaluation and conclusions

- 5 The PCR amplification strategy used has generated ALAD cDNA that matches the published sequence. It has convenient restriction sites at the ends for ease of manipulation into expression vectors, including an engineered BsrD I site just upstream of the ATG.

10 Example 9

Administration for rhPBGD, an animal study.

- Recombinant human Porphobilinogen Deaminase (rhPBGD) will be administered as an enzyme substitution treatment for patients diagnosed with Acute Intermittent Porphyria (AIP). rhPBGD will be administered by s.c or i.v injections. It is essential for the efficacy of the treatment, e.g. reduction of the toxic precursors porphobilinogen (PBG) and δ -aminolevulinic acid (ALA), that rhPBGD can enter the blood stream and remain biologically active.

20

Pharmacokinetics of rhPBGD

- To study the pharmacokinetics, wildtype healthy B6 mice were injected with rhPBGD. The content and enzyme activity of rhPBGD was followed in plasma from animals after different timepoints (0, 15, 30, 45 and 60 min). Each animal received one single injection of 50 μ g rhPBGD and three different routes of administration were used, i.v, i.p or s.c. The plasma levels of rhPBGD analysed by ELISA are shown in Figure 1. The conclusion from this data is that the half-life of rhPBGD following i.v injection is 20-30 min. Following i.p injection the maximal levels of rhPBGD was found after approximately 30 min. Also s.c injections of rhPBGD resulted in detectable levels of PBGD in plasma which shows that it is possible to use this route for administration. S.c injection did also result in a slow release of rhPBGD to plasma with maximal levels found in the last timepoint analysed (60 min).

- 38 shows plasma levels of rhPBGD following administration to mice. 50 μ g rhPBGD (2,3-2,8 mg/kg) were injected i.v, i.p or s.c to wildtype B6 mice. After different timepoints (0, 15,

30, 45 or 60 min respectively) mice were killed and plasma were prepared from blood obtained by heartpuncture. Levels of rhPBGD were analysed using an ELISA method where data are expressed as μg rhPBGD/ml plasma. Each point represents one animal.

5 In Figure 39 the same plasma samples were analysed using a PBGD enzyme activity assay to answer the question if the rhPBGD found in plasma is active. The pattern of the enzyme activity is very similar to Figure 38, showing that rhPBGD detected with the ELISA is also enzymatically active.

10 In conclusion it is observed that if rhPBGD is administered to mice using i.v, i.p or s.c injections the active enzyme is found in blood of those animals. The half-life of rhPBGD following i.v administration has also been shown to be approximately 20-30 min.

Figure 39 shows the PBGD enzymatic activity in plasma following rhPBGD administration to mice. 50 μg rhPBGD (2,3-2,8 mg/kg) were injected i.v, i.p or s.c to wildtype B6 mice. After different timepoints (0, 15, 30, 45 or 60 min respectively) mice were killed and plasma were prepared from blood obtained by heartpuncture. Enzymatic activity was determined in samples and are expressed as μg rhPBGD/ml plasma calculated by using a specific activity of rhPBGD of 14U/mg. Each point represents one animal.

20

Proof of concept study

Many AIP patients experience acute porphyric attacks. During these periods they excrete large amounts of the heme precursors PBG and ALA in urine. These precursors are
25 believed to be essential factors in the pathophysiology of the disease. An essential principle with the enzyme replacement therapy with rhPBGD is to metabolise and lower circulating PBG and ALA levels.

To analyse if rhPBGD can effect levels of PBG and ALA *in vivo*, a transgenic mouse was
30 used where the PBGD gene has partially been knocked-out (Lindberg, R. L. P. et al. Nature Genetics 12:195-199, 1996). This mouse-strain shows only 30% rest-activity of PBGD in liver. By treating these mice with phenobarbital one can induce a syndrome very similar to the human form of AIP. The AIP-attack can be followed in the animals by analysing secretion of ALA and PBG in urine (see Figure 40 and 41).

35

In this study all animals were treated with an increasing dose of phenobarbital for four days (day 0-4, 75-90 mg/kg and day i.p). One group of animals did at the same time also receive rhPBGD for seven days (day 0-7, 1,7-2,3 mg/kg and day i.p). The content of PBG and ALA were analysed in 24 h urine samples where levels are expressed as mmol/mol creatinine.

- 5 As seen in Figure 4 the rhPBGD treatment results in a lowering of urinary content of PBG and ALA as compared to animals treated with only phenobarbital (Figure 40). This data shows that rhPBGD, when given to mice with high levels of PBG and ALA in serum (acute AIP attack), can lower these levels as analysed by the urine content of this metabolites. No antibody formation against rhPBGD was seen in these animals when analysing at day 11
- 10 or at 2 weeks after that the rhPBGD treatment was stopped.

- The conclusion from this data is that rhPBGD can lower the levels of PBG and ALA in mice during an acute attack of porphyria. This may also indicate that clinical symptoms seen in AIP patients, which probably are induced by the high serum levels of PBG and ALA, may
- 15 be reversed by this treatment. Further studies are now on its way to confirm this data. It is also possible to increase the treatment period using rhPBGD in mice due to that no antibody formation was seen.

- Fig 40 shows the urinary content of PBG and ALA in AIP-mouse treated with
- 20 phenobarbital. Mice were treated with an increasing dose of phenobarbital for 4 days (day 0-4, 75-90 mg/kg and day i.p). PBG and ALA levels were analysed in 24-h urine samples and expressed as mmol/mol creatinine. Data from one representative animal are shown.

- Fig. 41 shows the urinary content of PBG and ALA in AIP-mouse treated with phenobarbital
- 25 and rhPBGD. Mice were treated with an increasing dose of phenobarbital for 4 days (day 0-4, 75-90 mg/kg and day i.p) and rhPBGD for 7 days (day 0-7, 1,7-2,3 mg/kg and day i.p). PBG and ALA levels were analysed in 24-h urine samples and expressed as mmol/mol creatinine. Data from one representative animal are shown.

30 Ongoing studies in mice

- Known clinical symptoms in AIP patient are different neurological symptoms such as pain in stomach and/or legs and arms and muscle weakness. To study these symptoms in the transgenic mice we also analysed the motorneuron function by different behavioural tests
- 35 such as rotarod and grip strength. Data shows that the transgenic AIP-mouse have

significantly lower activity in all behavioural tests as compared to wildtype controls. See examples of such data from the grip strength (Figure 5) and from rotarod (Figure 6). Motor neuropathy has also been described in the AIP-mice by Lindberg, R. L. P. et al. Journal of Clinical Investigation 103:1127-1134, 1999. We will now analyse if also the neurological disorders in the AIP-mouse can be reversed by the rhPBGD treatment, as a possible long-term treatment for patients.

Fig 42 shows the grip strength analysis in control and AIP-mice. Grip strength were determined using a grip strength meter (Ugo Basile) in heterozygous control animals (control 1, n=5), in wild type controls (control 2, n=5) and in AIP-transgenic mice (AIP, n=5).

Fig. 46 shows a rotarod analysis in control and AIP-mice. The rotarod analysis were determined using a rotarod treadmill (Ugo Basile) in wild type controls (control, n=5) and in AIP-transgenic mice (AIP, n=7).

Example10

Stability data for rhPBGD

20

Stability study 1 - Selection of formulation buffer

To find the best suitable formulation conditions for rhPBGD-His, the enzyme was formulated in a phosphate buffer containing mannitol and glycine as protein stabilizers. Different pH, ion strength and enzyme concentration were investigated, Table 47. The study was performed at 40°C and 75% relative humidity during 8 weeks.

Table 47. Formulations

Sample no.	pH	Ion strength (mM)	rhPBGD-His conc. (mg/ml)
1a, 2c, 3a	7,5	10	2
1b	7,5	10	8
2a	6,5	10	2
2b	7,0	10	2
2d	8,0	10	2

2e	8,5	10	2
3b	7,5	50	2
3c	7,5	100	2

The samples were sterile filtered and aliquots of 170 μ l were dispensed into 300 μ l glass vials with a Teflon/silicon crimp cap. Samples were collected for analysis on week 0, 1, 2, 4 and 8 and were analysed for enzyme activity (enzyme activity assay), protein concentration (HPLC) and degradation/aggregation (HPLC, SDS-PAGE and IEF).

The enzyme precipitated in all samples except for sample no. 2e (pH 8,5). Sample no. 1b (8 mg/ml) showed a higher precipitation rate than samples with lower enzyme concentration. This sample turned yellow after one week, all other samples turned yellow after 2 weeks. The HPLC chromatograms showed that a prepeak to the rhPBGD-His peak was formed during storage at 40°C, and that this prepeak increase with time. The peak for pure rhPBGD-His decreased with time. Figure 1, shows the amount enzyme calculated from the area under the peak in HPLC chromatograms.

The enzyme activity, Figure 45, decreased from 16 Units/ml (U/ml) to 2 U/ml in 8 weeks, for sample no. 1b the decrease in enzyme activity was more pronounced the first week, 40 U/ml to 10 U/ml, corresponding to the precipitation seen in Figure 44. The specific enzyme activity is shown in Figure 3. It seemed like high protein concentration was detrimental for the enzyme activity (sample no. 1b).

20

SDS-PAGE gels showed bands from aggregates as well as from scissoring after one week for all samples.

On the isoelectric focusing, IEF, gels four bands corresponding to the four catalytic forms of the enzyme (E, E1, E2 and E3) were seen on day 0. During storage at 40°C it seemed like the first band (E) was weakened and the second band (E1), which is more acidic was getting stronger. The number of more acidic bands increased over time. This was probably due to deamidation.

30 Conclusion

rhPBGD-His was not stable at 40°C. However, the only formulation buffer in which no visible precipitation of the enzyme was detected was no. 2e (pH 8,5). High concentration of

the enzyme seemed to be critical for both precipitation and enzyme activity. All samples turned yellow as well as precipitated, except for no. 2e that just turned yellow. The decreased enzyme activity can for example be a result of enzyme aggregation, scissoring and/or deamidation.

5

Stability study 2 - Storage temperature

This study was performed on rhPBGD without the five histidines. Stability study 1 showed that rhPBGD-His has its best stability in pH 8,5. Since rhPBGD has its isoelectric point about 0,5 pH units lower than the histidine tagged enzyme a formulation buffer with pH 8,0 was chosen in this study. The formulation buffer is described in Table 48.

10

Table 48. Formulation buffer for rhPBGD, 3,67mM phosphate buffer with mannitol and glycine, pH 8 and ion strength 10mM.

Excipient	Concentration (mM)
Na ₂ HPO ₄ •2H ₂ O	3,16
NaH ₂ PO ₄ •H ₂ O	0,51
Glycine	27
Mannitol	222

15

The samples were prepared as described in stability study 1. The study was performed over 12 weeks using two concentrations of rhPBGD, 10 mg/ml and 2 mg/ml. The examined temperatures were -20°C ± 5°C, 5°C ± 3°C and 25°C ± 2°C. The material was also tested for stability when freeze/thawed. The assays were the same as in stability study 1.

20

The enzyme concentration was relatively stable in this study compared to the stability study 1, Figure 47. Precipitation was only seen at 25°C and only for the high concentrated formulation. The precipitation appeared after one week, at the same time as a yellow colour. After eight weeks the high concentrated formulation stored at 5°C had turned yellow. For the 2 mg/ml formulation the yellow colour appeared only at 25°C, and then not until after eight weeks of storage. (The fluctuations seen in Figure 47 was probably due to variability in the dilution of the samples and integration of HPLC chromatograms.)

25

Regarding the enzyme activity there was no significant difference between the two concentrations or the temperatures -20°C and 5°C , Figure 48 and 49. The samples stored at 25°C showed a lower final enzyme activity.

- 5 The material used in this study showed at time 0 two bands at higher molecular weight than pure rhPBGD and one with lower molecular weight, on SDS-PAGE gels. This pattern did not change for any of the samples during the 12 weeks.

On the IEF gels there were two extra bands for samples stored at 5°C and 25°C , at lower pH than the original bands. These bands were more pronounced in the 10 mg/ml samples. For the samples stored at 5°C these bands appeared after 4 weeks and for the samples stored at 25°C after 1 week. This might be due to deamidations.

Conclusion

- 15 The rhPBGD formulation was best preserved at -20°C over 12 weeks. Samples stored at 5°C showed changes after 4 weeks on IEF gels, not recorded for -20 samples. Except for this 5°C seemed acceptable for storage of the rhPBGD formulation over 12 weeks.

Stability study 3 - Simulation of toxicology selection study

20

This 12 weeks study was performed using the same formulation buffer as in stability study 2, Table 48. The purpose of this study was to certify that the rhPBGD should be stable during the animal selection study. The formulation for the 58 days long selection study will be kept frozen (-20°C) until the day before use. At this point it will be thawed at 5°C over 25 night. During use it will probably be stored at room temperature (25°C). In this study a formulation with a concentration of 10 mg/ml of rhPBGD will be used.

The samples were sterile filtered and aliquots of 400 μl sample were dispensed into 3 ml glass vials with rubber stopper. The vials were flushed with nitrogen and subsequently 30 capped with a plastic seal. A formulation with 10 mg/ml of rhPBGD was used, and its stability at $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$ for 12 weeks, at $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$ for 4 weeks and at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 weeks was examined. The protein concentration in the samples was in this study measured using BCA, Figure 50.

The variability in protein concentration seen in the HPLC measurements was reduced using this method. However, the variability in the data seen in Figure 50 was probably due to variability in dilution of the samples.

- 5 This high concentrated formulation was from origin yellowish in colour. After 8 weeks, the formulation had become yellow. No precipitation was seen after 8 weeks (the study is not finished).

- The enzyme activity was stable during the investigated time period at each temperature,
10 Figure 51. This was true even for the specific enzyme activity, Figure 52. There were some fluctuations, however these were probably assay related.

- The material used in this study showed from origin two bands at higher molecular weight than pure rhPBGD and one with lower molecular weight, on SDS-PAGE gels. One weak
15 extra band at even lower molecular weight appeared after 2 weeks at 25°C and after 4 weeks at 5°C. This band had not appeared after 8 weeks at -20°C.

- The samples showed at time 0 two extra bands on the IEF gels. These were more acidic than the E3 band, however very weak,. For samples stored at 5°C and at 25°C the intensity
20 of these bands increased over time.

Conclusion

- The rhPBGD will stay stable during the selection study if treated as described. Thus, rhPBGD is stable at -20°C for 8 weeks, and except for deamidation at 5°C for 4 weeks and
25 at 25°C for 2 weeks. Note, that this study was performed using nitrogen in the test vials.

References:

- Anderson P. M. and R. J. Desnick. 1979, Purification and Properties of Uroporphyrinogen I Synthase from Human Erythrocytes: Identification of Stable Enzyme-Substrate Intermediates *The Journal of Biological Chemistry* 255(5) :1993-99
- Andersson, Christer, Thesis, 1997, ISBN 91/7191/280/0, pp. 22-23
- Amann, E., Brosius, J., and Ptashne, M. 1983, Vectors bearing a hybrid trp-lac promoter useful for regulated expression of cloned genes in *Escherichia coli*. *Gene* 25(2-3):167-178
- Awan S.J. et al. 1997, Reconstitution of the Holoenzyme Form of *E. coli* Porphobilinogen Deaminase from Apoenzyme with Porphobilinogen and Preuroporphyrinogen: A Study Using Circular Dichroism Spectroscopy *Biochemistry* 36 (30) :9273-82
- Gold, L. and Stormo, G.D. 1990, High-level translation initiation. *Methods Enzymol.* 185:89-93
- Grandchamp B. et al. 1987, Tissue-specific expression of porphobilinogen deaminase. Two isoenzymes from a single gene. *Eur J Biochem.* Jan;162(1):105-10
- Grandschamp B. et al. 1996, *J. of Gastroenterology and Hepatology* 11, 1046-1052
- Herrick A.L. et al. 1989, *Lancet* 1, 1295-1297
- Jeans J. et al. 1996, *American J. of Medical Genetics*, 65, 269-273
- Jordan P.M., S.D. Thomas and M.J. Warren. 1988, Purification, crystallization and properties of porphobilinogen deaminase from a recombinant strain of *Escherichia coli* K12. *Biochem. J.* 254: 427-435
- Jordan, P.M. The biosynthesis of uroporphyrinogen III: mechanism of action of porphobilinogen deaminase. In: *The biosynthesis of the tetrapyrrole pigments* 1994 Wiley, Chister (Ciba Found Symp 180), p70-96

- Lambert R. et al. 1994, Structural studies on porphobilinogen deaminase. In: The biosynthesis of the tetrapyrrole pigments. Wiley, Chichester (Ciba Found Symp 180), p97-110
- 5 Lewis, L.A., Li, K.B., Gousse, A., Pereira, F., Pacheco, N., Pierre, S., Kodaman, P., and Lawson, S. 1991, Genetic and molecular analysis of spontaneous respiratory deficient (res-) mutants of *Escherichia coli* K-12. *Microbiol.Immunol.* 35(4):289-301
- Lithner F. et al. 1984, *Acta.Med.Scand.* 215, 271-274
- 10 Louie, G.V. et al. 1996, The three-dimensional structure of *Escherichia coli* porphobilinogen deaminase at 1.76-A resolution *Proteins* 25(1):48-78
- Makrides, S.C. 1996, Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiol.Rev.* 60(3):512-538
- 15
- Maniatis T., E.F. Fritsch, J. Sambrook. *Molecular Cloning (A laboratory Manual)* Cold Spring Harbor Laboratory. 1982
- 20 Miller et al. 1989, *BioTechniques* 7, 980-990
- Miyagi K. et al. 1979, Uroporphyrinogen I synthase from human erythrocytes: Separation, purification, and properties of isoenzymes. *Proc. Natl. Acad. Sci.* 76:12 6172-76
- 25 Morrison D.A. 1979, Transformation and preservation of competent bacterial cells by freezing. *Methods Enzymol.* 68, 326-331.
- Mustajoki et al. 1989, *Sem. Hematol.* 26, 1-9
- 30 Raich N. et al. 1986, Molecular cloning and complete primary sequence of erythrocyte porphobilinogen deaminase. *Nucleic Acids Research* 14(15): 5955-67
- Raich N. et al. 1986, *Nucleic. Acid. Res.* 14, 5955-5968

Saiki R.K. et al. 1985, Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science, 20;230(4732):1350-4.

Sassa S. 1996, Blood Review, 10, 53-58

5

Shoolingin-Jordan P.M., M.J. Warren and S.J. Awan, 1997, Dipyrrromethane Cofactor Assembly of Porphobilinogen Deaminase: Formation of Apoenzyme and Preparation of Holoenzyme. Methods in Enzymology 281: 317-327

10 Stephens P.E. et. al. Biochem J. 248, 1-11, 1987

Strand et al. 1970, Proc. Natl. Acad. Sci. 67, 1315-1320

Tishler P.V. et al. 1985, Am.J.Psychiatry 142,1430-1436

15

Waldenström J. 1937, Acta.Med. Scand. Suppl.82

Welland F.H. et al. 1964, Metabolism, 13, 232

20 Wetterberg L. 1967, Svenska bokförlaget Nordstedt, Stockholm

Wetterberg L. 1976, In Doss M. Nowrocki P. eds. Porphyrrias in Human Disease. Reports of the discussion. Matgurg an der Lahn, 191-202

25 Yeung L. et al. 1983, Q J. Med 52, 92-98

Yoo H.W. et al. 1993, Hydroxymethylbilane synthase: complete genomic sequence and amplifiable polymorphisms in the human gene. Genomics Jan; 15(1):21-9

30 References for the method relating to example 7 (A)

(1) Jordan P.M. et al (1988) Purification, crystallisation and properties of porphobilinogen deaminase from a recombinant strain of Escherichia coli K12.Biochem. J 254, 427-435

(2) Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685

35

- (3) Novex (1997) NuPAGE Electrophoresis System - Instruction Booklet.
- (4) Oort P.J. Purification of Recombinant HIS-Tagged Human Erythropoietic Porphobilinogen Deaminase (rPBGD-HIS) Report number 3
- (5) Pierce Instructions (1997) BCA Protein assay Kit, version 4/1997
- 5 (6) Shoolingin-Jordan P.M. et al. (1977) Dipyrromethane cofactor assembly of porphobilinogen deaminase: Formation of apoenzyme and preparation of holoenzyme. *Methods in Enzymology* 281, 317-327
- (7) Technical Report Master and Working Cell Bank Development, HemeBiotech Internal no: 01000043
- 10 (8) Development of expression system for recombinant human porphobilinogen deaminase, HemeBiotech Internal no: 0199003

15 References to example 8 Cloning of ALAD

- Kaya AH, Plewinska M, Wong DM, Desnick RJ, Wetmur JG. Human delta-aminolevulinate dehydratase (ALAD) gene: structure and alternative splicing of the erythroid and housekeeping mRNAs. *Genomics* 19[2], 242-248. 1994.
- 20 Wetmur JG, Bishop DF, Ostasiewicz L, Desnick RJ. Molecular cloning of a cDNA for human delta-aminolevulinate dehydratase. *Gene* 43[1-2], 123-130. 1986.

25

CLAIMS

1. A method for treatment or prophylaxis of disease caused by deficiency, in a subject, of one or more enzymes belonging to the heme biosynthetic pathway, the method comprising
- 5 administering, to the subject, an effective amount of one or more catalysts which is/are said enzyme(s), or an enzymatically equivalent part or analogue thereof, optionally in combination with gene therapy of a mutation related to the a disease of the heme biosynthetic pathway
- 10 2. A method according to claim 1, wherein the disease is selected from the group consisting of
- acute intermittent porphyria (AIP),
ALA deficiency porphyria (ADP),
Porphyria cutanea tarda (PCT),
- 15 Hereditary coproporphyria (HCP),
Harderoporphyria (HDP),
Variegata porphyria (VP),
Congenital erythropoietic porphyria (CEP),
Erythropoietic protoporphyria (EPP), and
- 20 Hepatoerythropoietic porphyria (HEP).
3. A method according to claim 1, wherein the catalyst is one or more enzymes selected from the group consisting of
- delta-aminolevulinic acid synthetase,
- 25 delta-aminolevulinic acid dehydratase (ALAD),
porphobilinogen deaminase (PBGD),
uroporphyrinogen III cosynthetase,
uroporphyrinogen decarboxylase,
coproporphyrinogen oxidase,
- 30 protoporphyrinogen oxidase, and
ferrochelataase,
or an enzymatically equivalent part or analogue thereof.

4. A method according to claim 1, wherein the disease is AIP and the enzyme is PBGD or an enzymatically equivalent part or analogue thereof, preferable in combination with ALAD
5. A method according to claim 1, wherein the catalyst is a recombinant form of the
- 5 enzyme belonging to the heme biosynthetic pathway or of the enzymatically equivalent part or analogue thereof.
6. A method according to claims 1, wherein the catalyst is administered by a route selected from the group consisting of the intravenous route, the intraarterial route, the
- 10 intracutaneous route, the subcutaneous route, the oral route, the buccal route, the intramuscular route, the anal route, the transdermic route, the intradermal route, and the intratechal route.
7. A method according to any of claim 1, wherein the catalyst is formulated in an isotonic
- 15 solution, such as 0.9% NaCl and 10-50 mM Sodium phosphate pH 6.50 to 8 or Sodium phosphate, glycine, mannitol or the corresponding potassium salts.
8. A method according to any of claim 7, wherein the catalyst is lyophilised.
- 20 9. A method according to any of claim 8, wherein the catalyst is sterile filtered.
10. A method according to any of claim 1, wherein the catalyst is formulated as lipid vesicles comprising phosphatidylcholine or phosphatidylethanolamine or combinations thereof.
- 25 11. A method according to any of claim 1, wherein the catalyst is incorporated into erythrocyte ghosts.
12. A method according to any of claim 1, wherein the catalyst is formulated as a sustained
- 30 release formulation involving biodegradable microspheres, such as microspheres comprising polylactic acid, polyglycolic acid or mixtures of these.

13. A method according to any of claim 1, wherein the catalyst is lyophilized in a two-compartment cartridge, where the catalyst will be in the front compartment and water for reconstitution in the rear compartment.
- 5 14. A method according to claim 13, wherein the two compartment cartridge is combined with an injection device to administer the catalyst either by a needle or by a needle-less (high pressure) device.
- 15 15. A method according to any of claim 1, wherein the catalyst is formulated in a physiological buffer containing an enhancer for nasal administration.
16. A method according to claim 1, wherein the catalyst is formulated as an oral formulation containing lipid vesicles, such as those comprising phosphatidylcholine, phosphatidylethanolamine, or sphingomyeline, or dextrane microspheres.
- 15 17. A method according to claim 1, wherein the catalyst is formulated so as to enhance the half-life thereof in the subject's bloodstream.
18. A method according to claim 17, wherein the catalyst has a polyethylene glycol coating.
- 20 19. A method according to claim 17, wherein the catalyst is complexed with a heavy metal.
20. A method according to claim 1, wherein the catalyst is an enzymatically equivalent part or analogue of the enzyme and exerts at least part of its enzymatic activity intracellularly
- 25 upon administration to the subject.
21. A method according to claim 20, wherein the catalyst is a small artificial enzyme or an organic catalyst which can polymerize porphobilinogen to hydroxymethylbilane
- 30 22. A method according to claim 1, wherein the catalyst is said enzyme formulated in such a manner that it exerts at least part of its enzymatic activity intracellularly upon administration to the subject.

23. A method according to claim 22, wherein the catalyst is tagged with specific carbohydrates or other liver cell specific structures for specific liver uptake.
24. A method according to claim 1, wherein the catalyst exerts substantially all its enzymatic activity extracellularly in the bloodstream.
25. A method according to claim 24, wherein the enzymatic activity of the catalyst on its relevant heme precursor results in a metabolic product which 1) either moves into the intracellular compartment and is converted further via the remaining steps of the heme biosynthetic pathway or 2) is excreted from the subject via urine and/or faeces.
26. A method according to claim 1, wherein the catalyst has been prepared by a method comprising
- a) introducing, into a suitable vector, a nucleic acid fragment which includes a nucleic acid sequence encoding the catalyst;
 - b) transforming a compatible host cell with the vector;
 - c) culturing the transformed host cell under conditions facilitating expression of the nucleic acid sequence; and
 - d) recovering the expression product from the culture and optionally subjecting the expression product to post-translational processing, such as in vitro protein refolding, enzymatic removal of fusion partners, alkylation of amino acid residues, and deglycosylation, so as to obtain the catalyst.
27. A method according to claim 1, wherein the catalyst has been prepared by liquid-phase or solid-phase peptide synthesis.
28. A method according to claim 26 of the preceding claims, wherein the catalyst is free from any other biological material of human origin.
29. A method according to any claim 1, wherein the catalyst is administered at least once a day, such as 2, 3, 4, or 5 times daily.

30. A method according to claim 29 wherein the daily dosage is in the range of 0.01 – 1.0 mg/kg body weight per day, such as in the range of 0.05 – 0.5 mg/kg body weight per day.

31. A method according to claim 29, wherein the daily dosage is about 0.1 mg per kg body weight per day.

32. A method according to claims 1 wherein the catalyst is a recombinant form of the enzyme.

10 33. A method according to claim 32 wherein the catalyst is recombinant human PBGD based on any of Seq. ID NO 3 (clone PBGD 1.1) and Seq. ID NO 4 (non-erythro PBGD 1.1.1).

34. A method for treating a patient having a mutation in the PBGD gene causing an
15 enzyme defect, comprising the use of a human PBGD cDNA sequence of either non-erythropoietic form or erythropoietic form according to whether the tissue in which PBGD should be expressed is in cells of erythroid origin or in other cells, and transfection of the patient with the relevant cDNA.

20 35. The method according to claim 34, wherein the enzyme deficiency is selected from enzyme deficiencies resulting in a disease selected from Acute Intermittent Porphyria, (AIP), ALA deficiency porphyria (ADP), Porphyria cutanea tarda (PCT), Hereditary coproporphyria (HCP), Harderoporphyria (HDP), Variegata porphyria (VP), Congenital erythropoietic porphyria (CEP), Erythropoietic protoporphyria (EPP), and Hepatoerythropoietic porphyria
25 (HEP).

36. The method according to claim 35 wherein the disease is Acute Intermittent Porphyria, (AIP).

30 37. The method according to claim 34, wherein the human PBGD cDNA sequence is selected from Seq. ID NO 3 (clone PBGD 1.1) and Seq. ID NO 4 (non-erythro PBGD 1.1.1).

38. The method according to any of claims 34 wherein the transfection is by use of a vector selected from adenovirus, retrovirus and associated adenovirus.

39. The method according to claim 34 wherein the PBGD transfection of the patient
5 (erythropoietic and/or non-erythropoietic cells) results in substantially normal PBGD activity measured as a normalisation in urinary and/or serum levels of delta-aminolevulinic acid (ALA) and porphobilanogen (PBG) compared to the levels before treatment or to a reduction in the frequency of attack of symptoms.

10 40. A method of gene therapy treatment of patients with Acute Intermittent Porphyria (AIP) by a correction of one of the specific point mutations identified causing AIP by use of chimeraplasty gene repair.

41. The method according to claim 40 comprising a delivery system for transfection which
15 is by use of non-viral vectors formulated in a vehicle preparation comprising one or more components selected from cationic phospholipids, phospholipids, phospholipids mixed with neutral lipids, lictosylated PEI, liposomes liposomes comprising mixtures of natural phospholipids and neutral lipids.

20 42. A method according to claim 40 wherein the mutation is selected from Table A.

43. A catalyst which is an enzyme of the heme biosynthetic pathway or an enzymatically equivalent part or analogue thereof, for use as a medicament.

25 44. A catalyst according to claim 43, which is recombinant human PBGD based on any of Seq. ID NO 3 (clone PBGD 1.1) and Seq. ID NO 4 (non-erythro PBGD 1.1.1).

45. An expression plasmid pExp1-M2-BB as shown in Seq. ID NO 1 for use in the expression of rhPBGD in *E. coli*.

30

46. A DNA fragmet, EcoR I - Hind III linear fragment as shown in Seq. ID NO 2, capable of obtaining *hemC*-deletion in a host.

47. A production strain of rhPBGD obtained by use of the DNA fragmet, EcoR I - Hind III linear fragment as shown in Seq. ID NO 2 to obtain *hemC*-deletion in the host JM105-H-R6-C by homologous gene replacement and transforming the resulting strain with the expression plasmid pExp1-M2-BB to yield the final production strain PBGD which is free
5 from production of PBGD of non human origin (Accsession No 12915).

48. A method for the preparation of rhPBGD by a method comprising

- a) introducing, into a suitable vector, a nucleic acid fragment which includes a nucleic acid
10 sequence encoding PBGD;
- b) transforming a compatible host cell with the vector;
- c) culturing the transformed host cell under conditions facilitating expression of the nucleic acid sequence;
- d) recovering the expression product from the culture.

15

49. A method according to claim 48 further comprising a fermentation step.

50. A method according to claim 48 further comprising a purification step.

20 51. A method according to claim 50 wherein the purification is performed wiht a His-Tag (rhPBGD-His).

52. A method according to any of claims 1-52 wherein the patient is treated subcutaneously with the catalyst.

25

53. 43. A catalyst which is an enzyme of the heme biosynthetic pathway or an enzymatically equivalent part or analogue thereof, for use as a medicament.

54. Use of one or more catalyst according to any of the preceding claims for the
30 preparation of a medicament for treating a disease related to a disease in the heme biosynsthetic pathway.

55. Use according to claim 54 wherein the disease is selected from the group comprising of

acute intermittent porphyria (AIP),

ALA deficiency porphyria (ADP),

5 Porphyria cutanea tarda (PCT),

Hereditary coproporphyria (HCP),

Harderoporphyria (HDP),

Variegata porphyria (VP),

Congenital erythropoietic porphyria (CEP),

10 Erythropoietic protoporphyria (EPP), and

Hepatoerythropoietic porphyria (HEP).

56. A rhPBGD having a stability of at least 6 weeks at -20°C, such as for at least 7 weeks, preferable for 8 weeks.

15

57. A rhPBGD having a stability resulting in a decrease in activity of less than 10% per month, such as less than 5%.

1/49

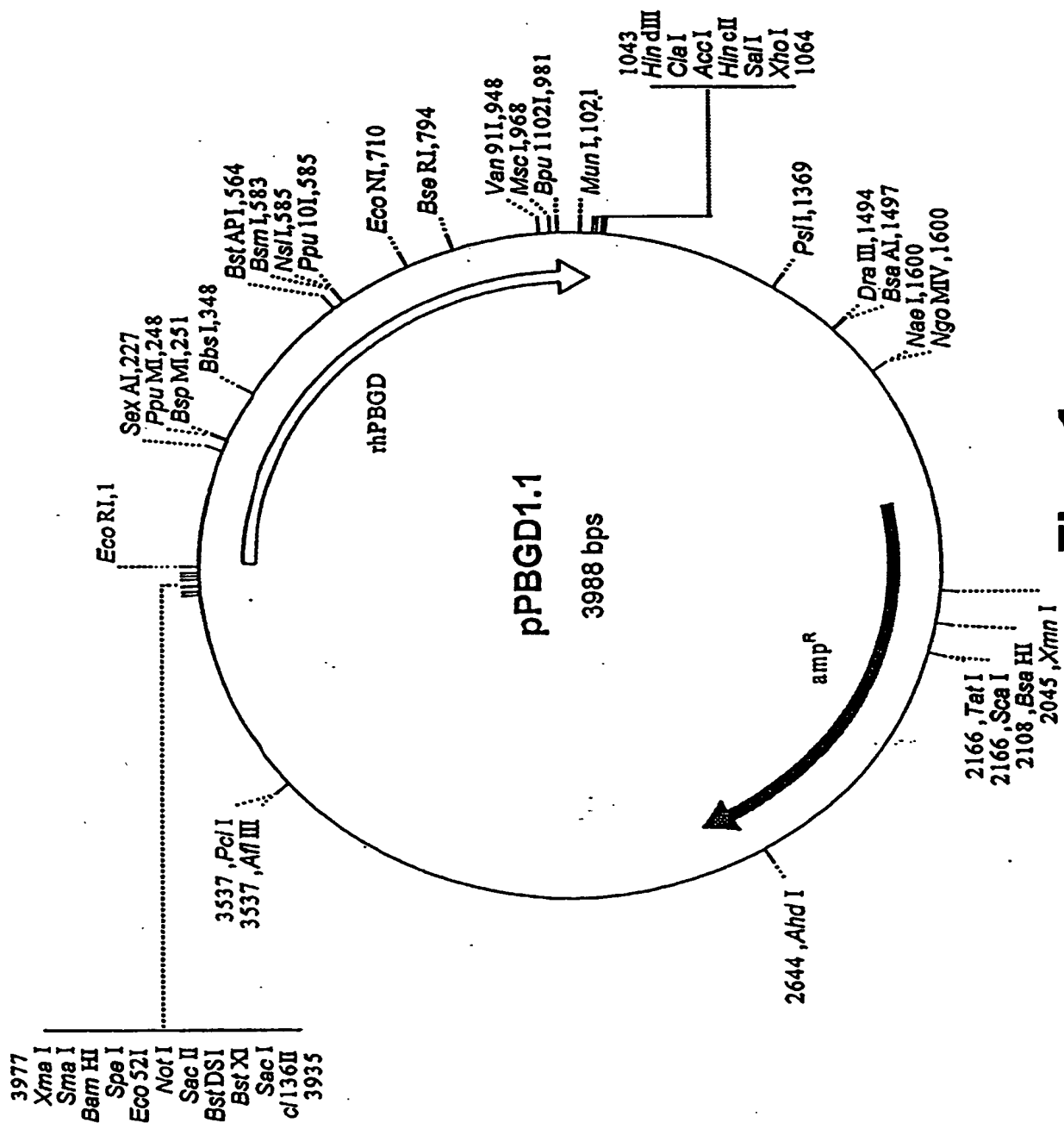


Fig. 1

2/49

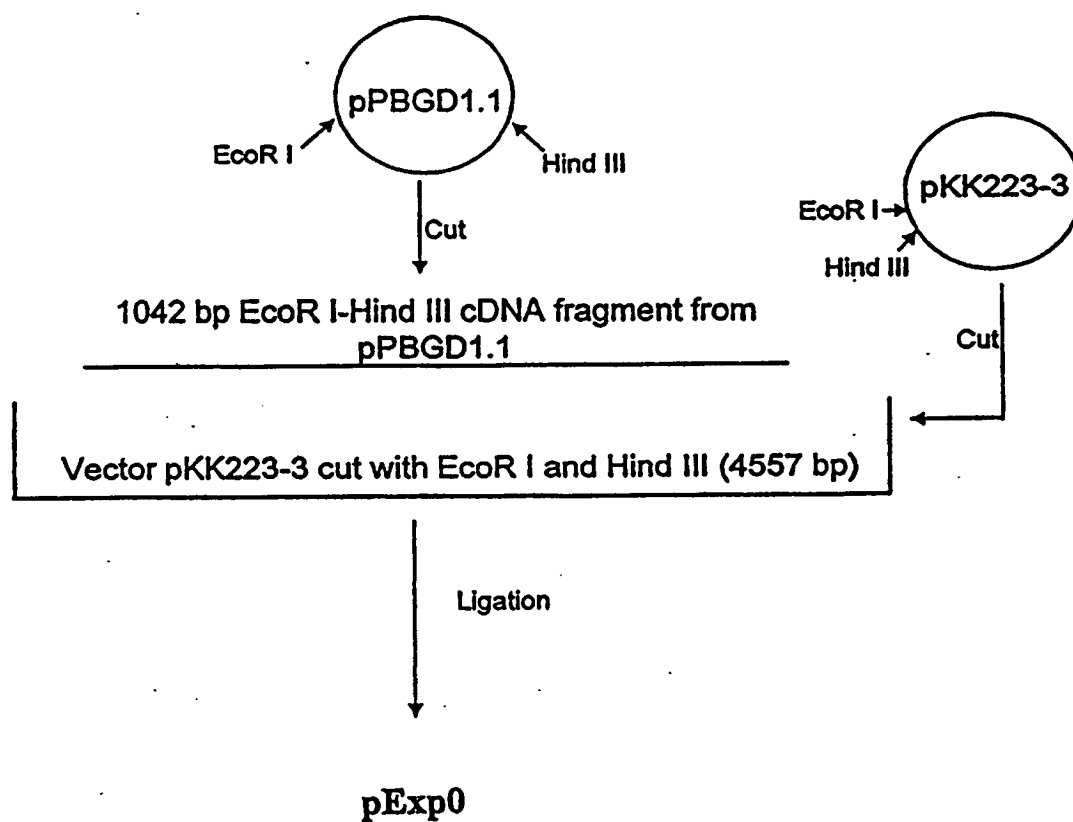


Fig. 2

3/49

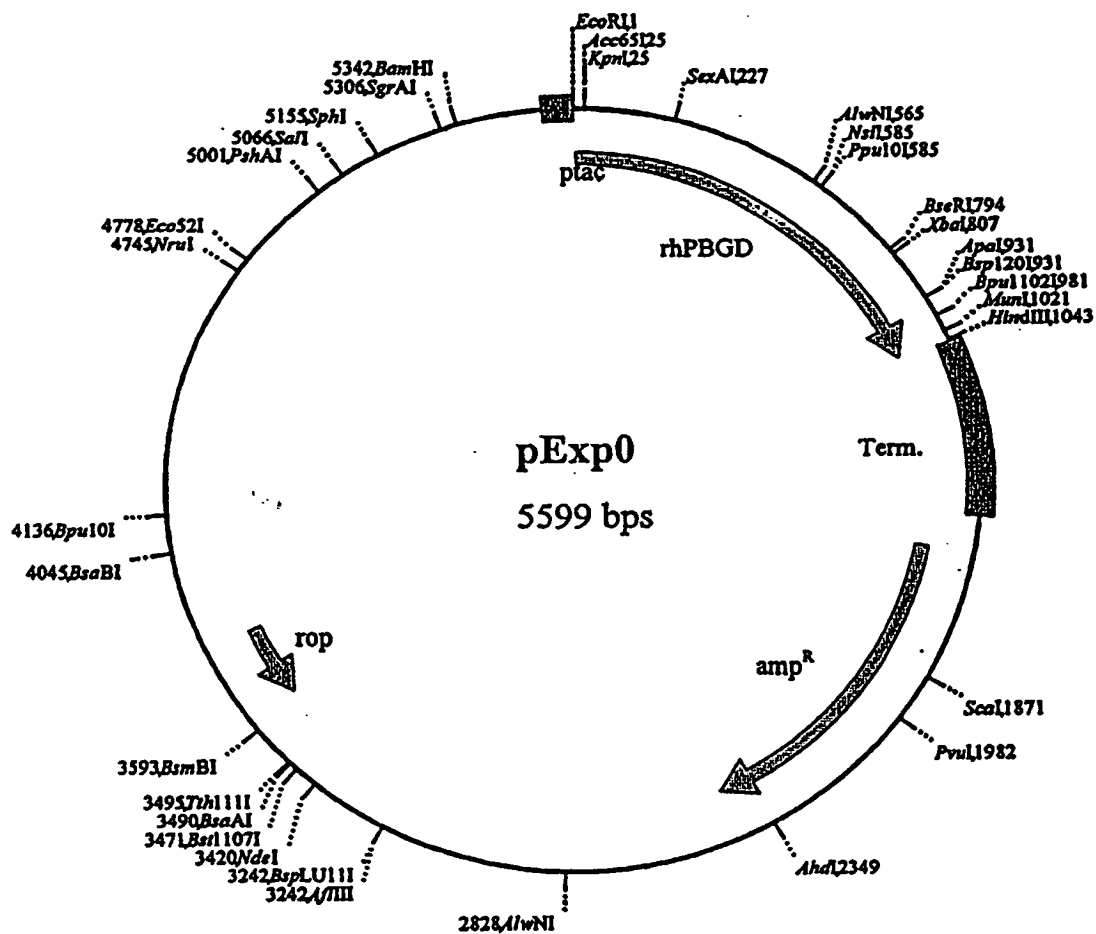


Fig. 3

4/49

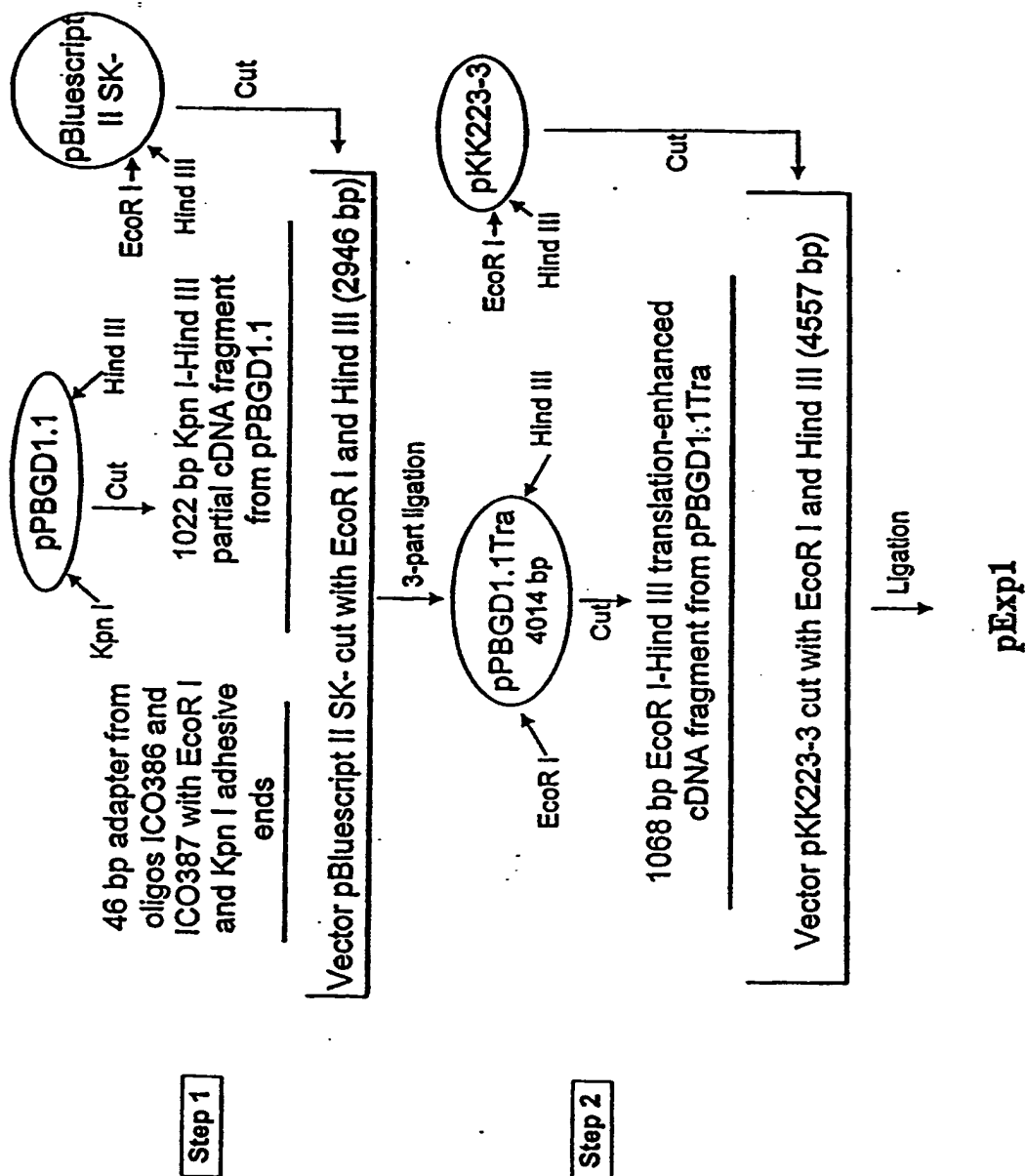


Fig. 4

5/49

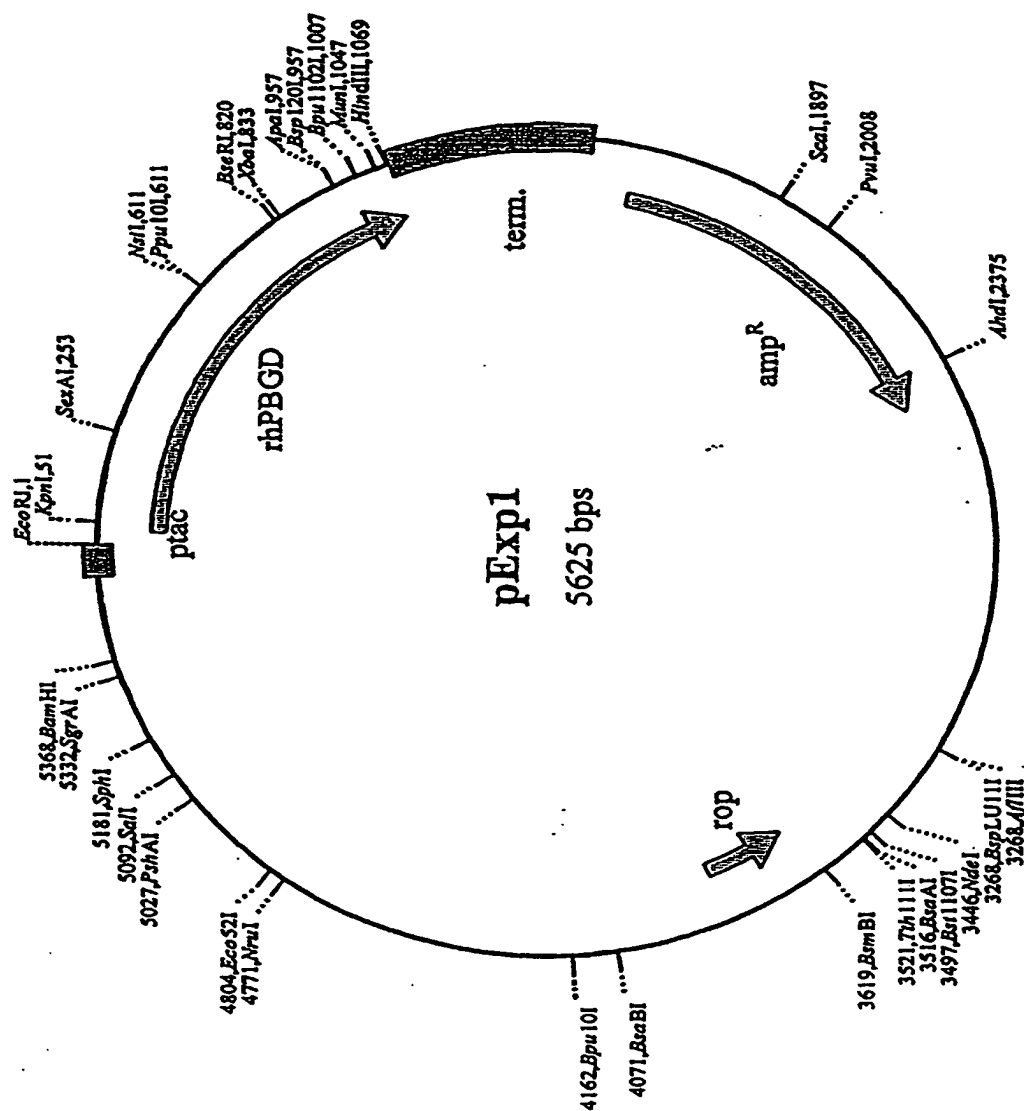
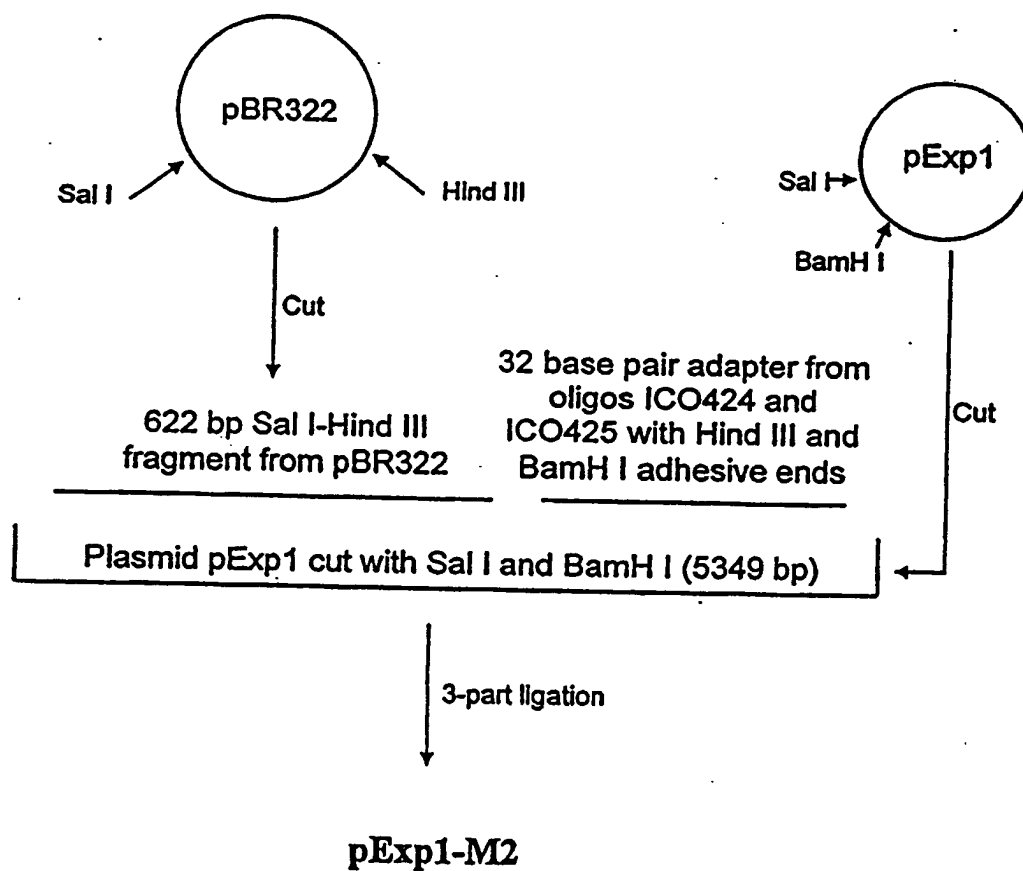


Fig. 5

6/49

**Fig. 6**

7/49

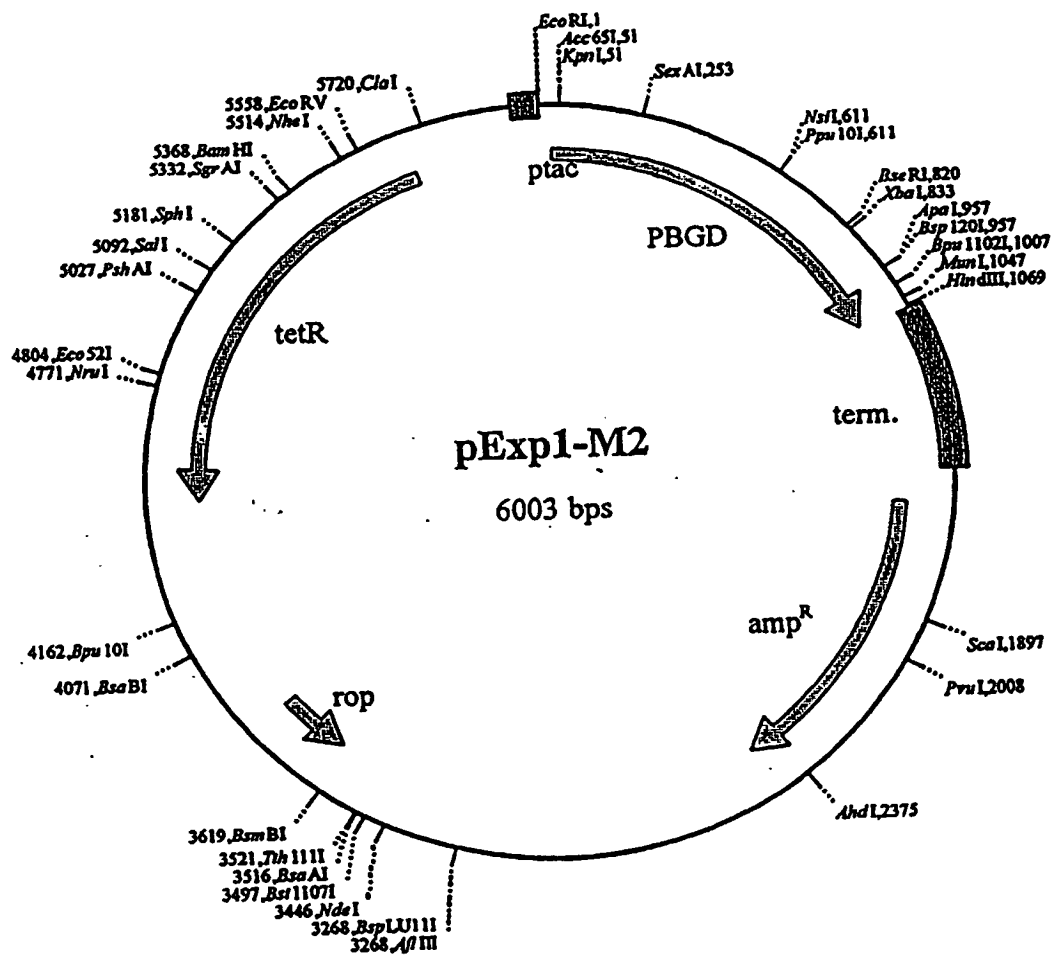
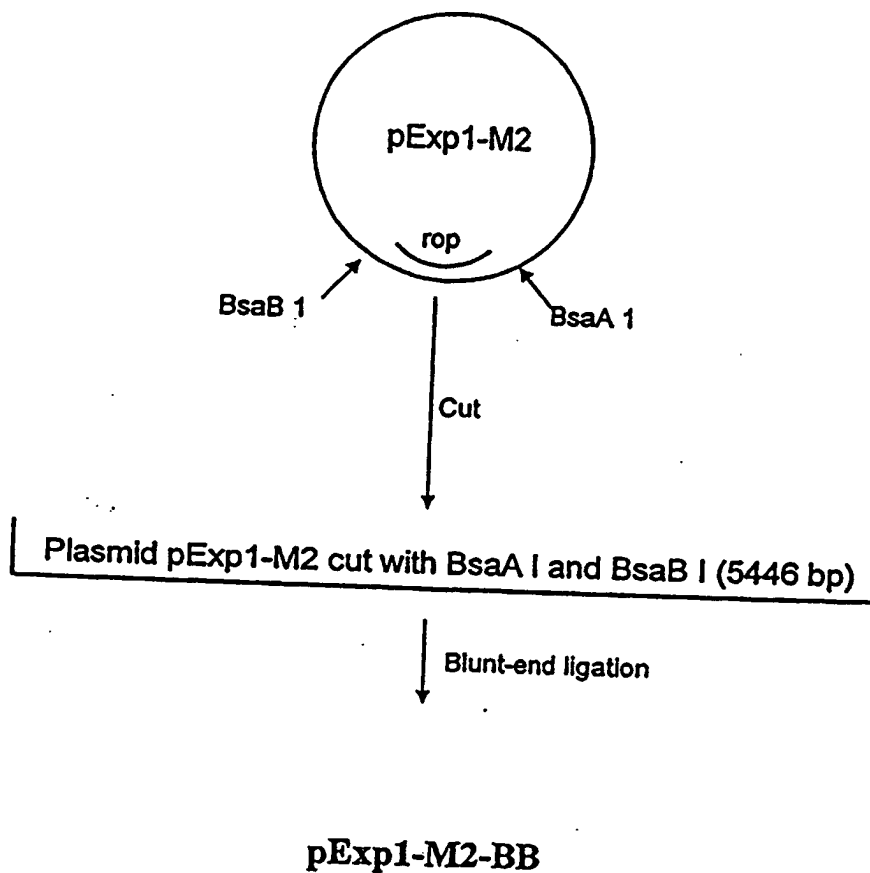
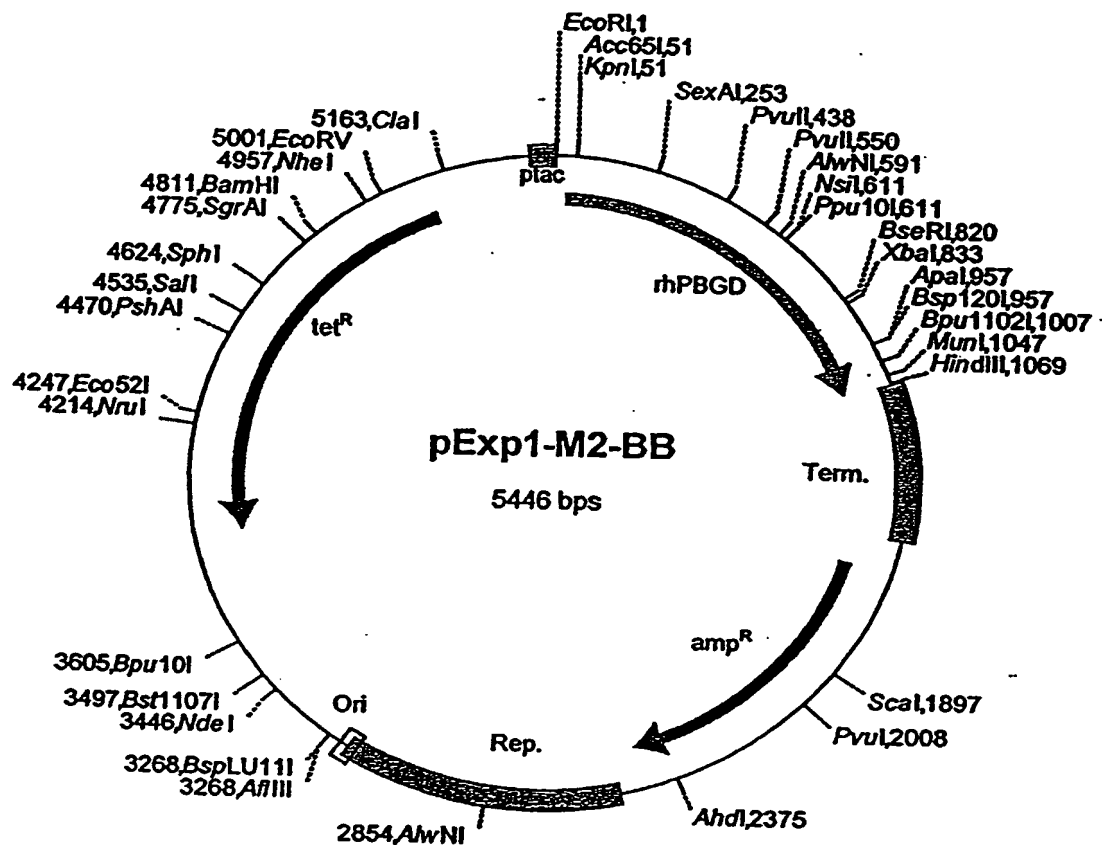


Fig. 7

8/49

**Fig. 8**

9/49



PLASMID FEATURES:

Start	End	Name
33	1067	rhPBGD (open reading frame)
1593	2453	amp ^R (open reading frame)
5105	3915	tet ^R (open reading frame)
5376	1	ptac (promoter)
1075	1501	Term. (terminator region)
3214	2519	Rep. (replication region)
3214		Ori (origin of replication)

Fig. 9

10/49

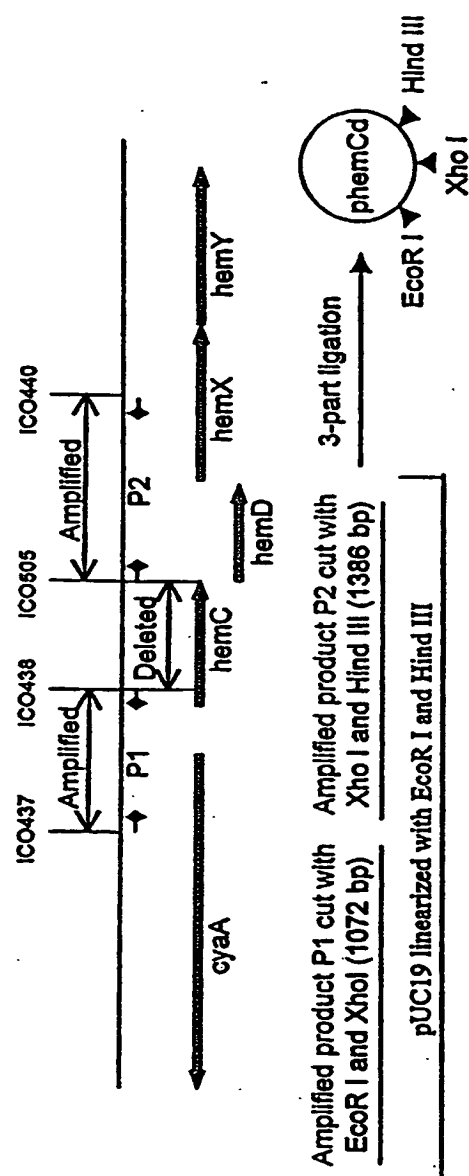


Fig. 10

11/49

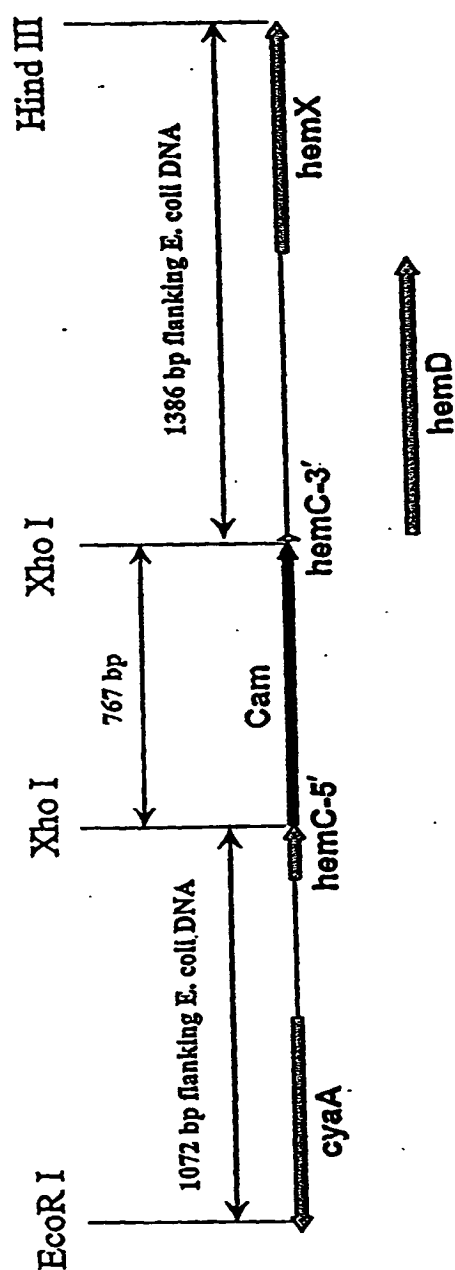


Fig. 11

12/49

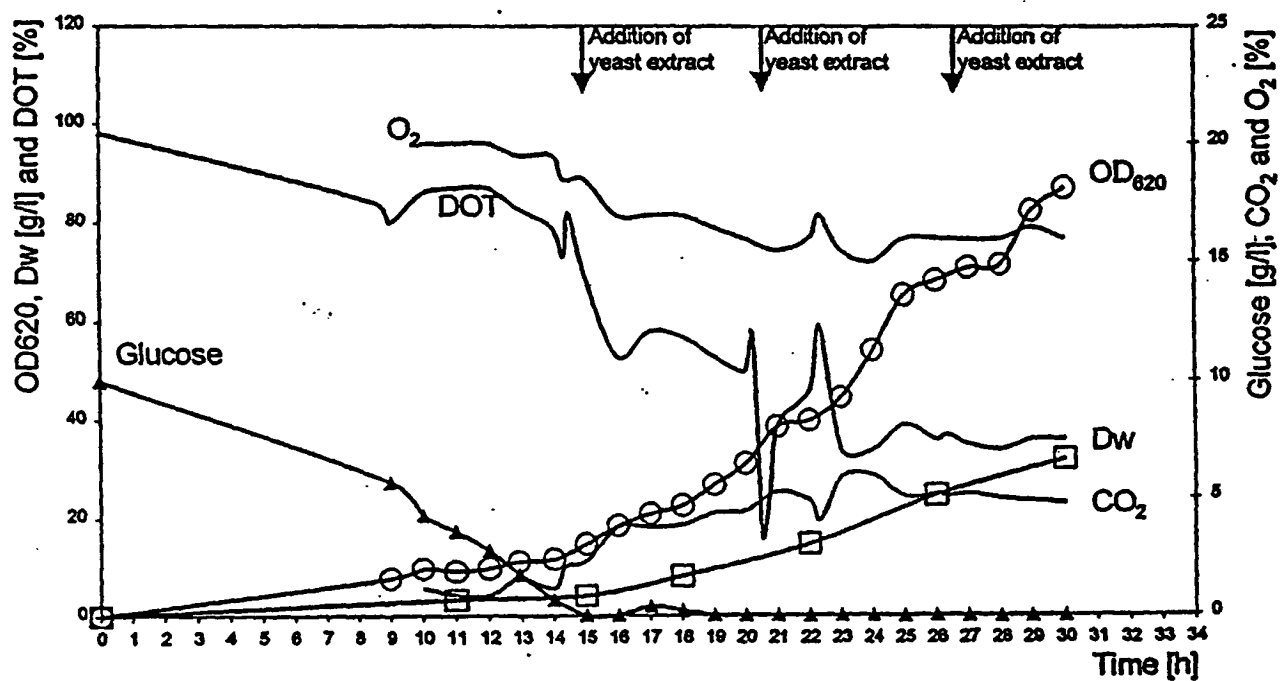


Fig. 12

13/49

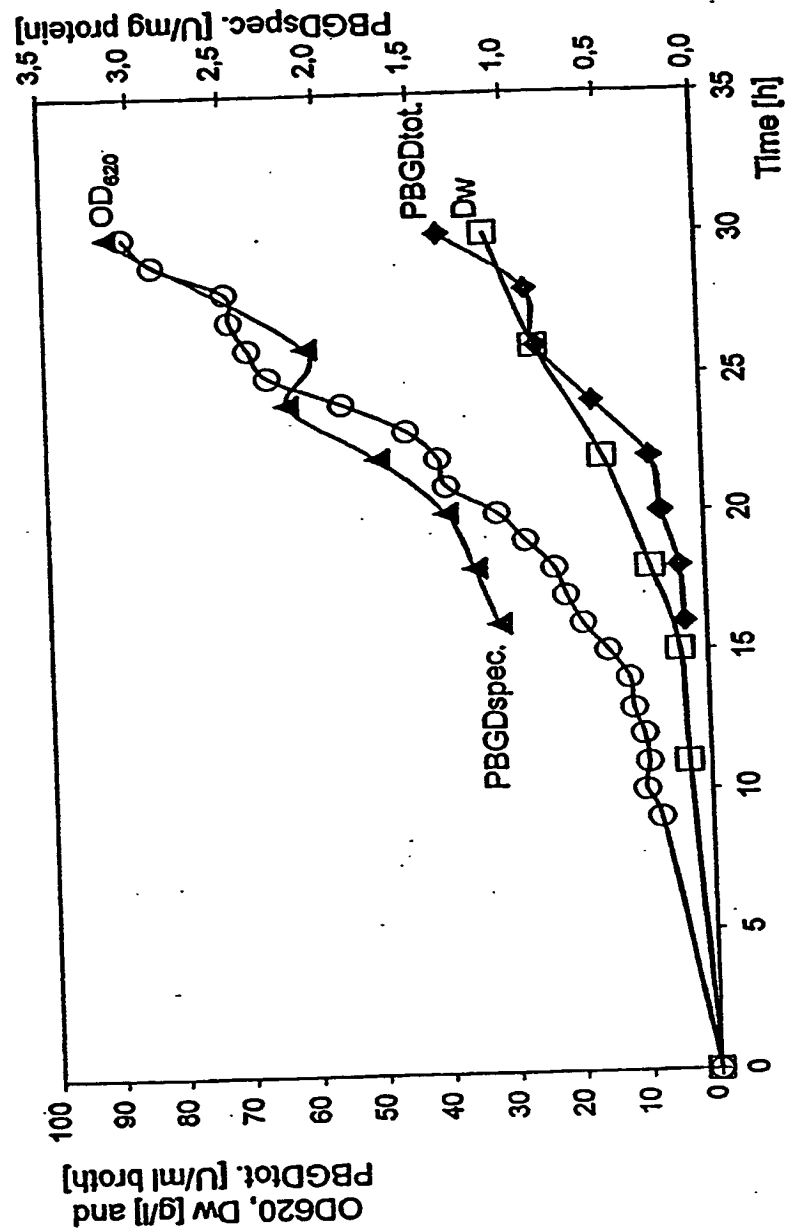
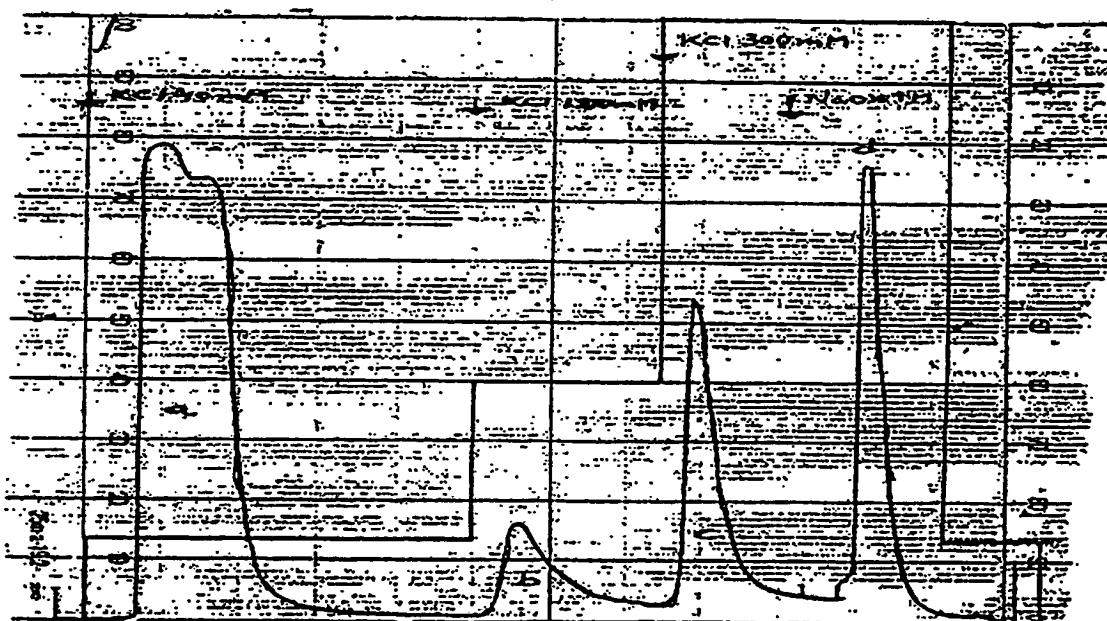


Fig. 13

Fig. 13

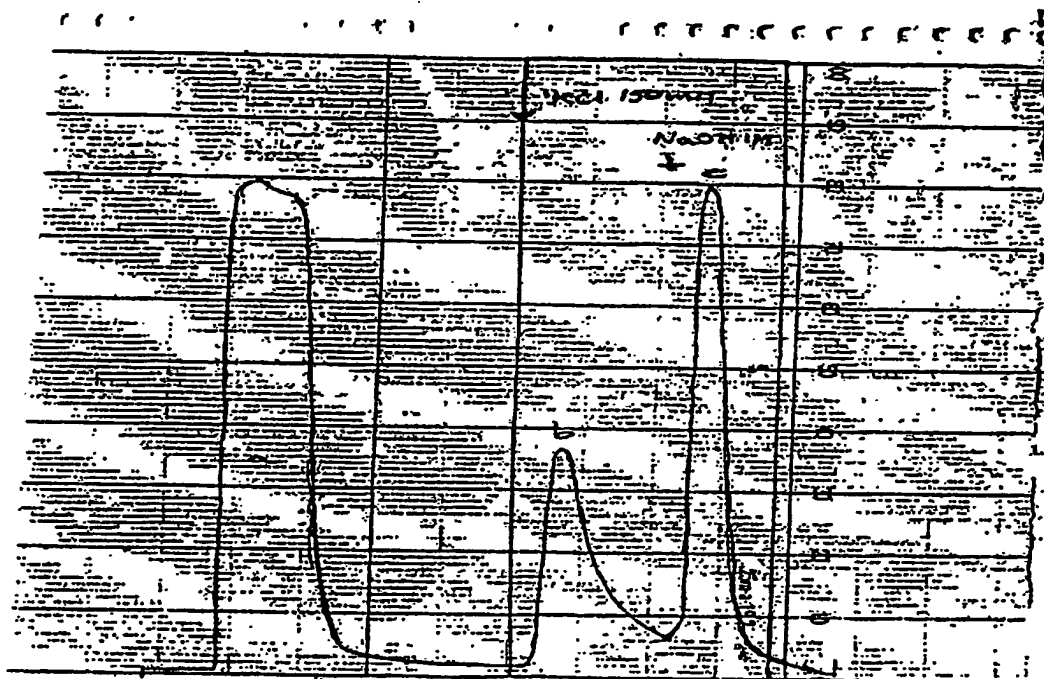
14/49



Chromatography on DEAE-Sepharose FF (DEAE1). Peak a flow through the gel 40 mM KCl, Peak b 120 mM KCl Peak c 300 mM KCl Peak d NaOH 1 M.

Fig. 14

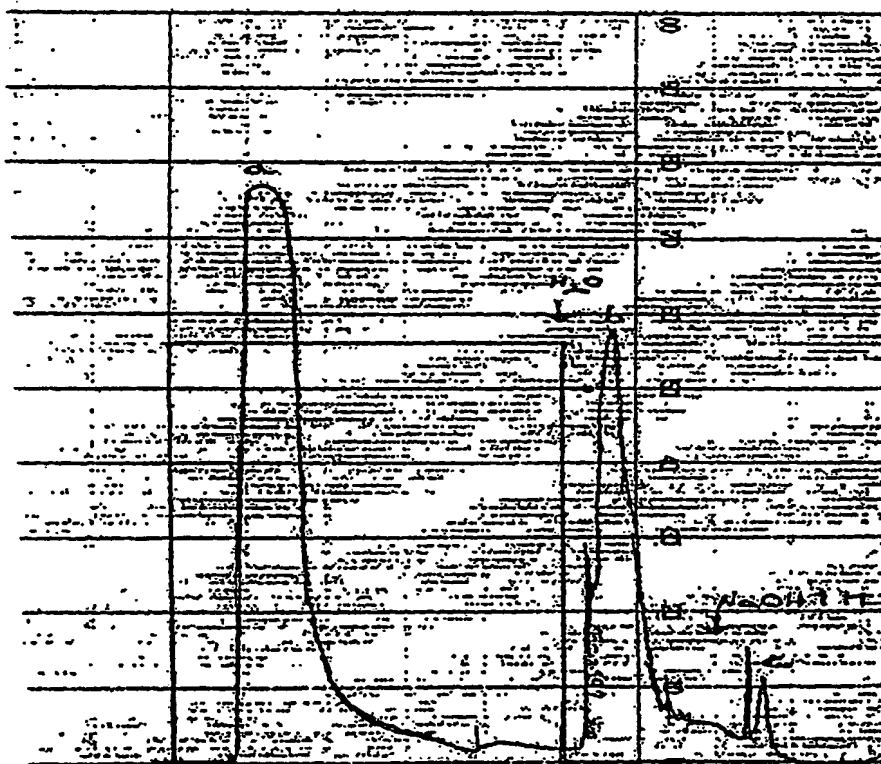
15/49



Chromatography on DEAE-Sepharose FF (DEAE2). Peak a flow through the gel 40 mM KCl, Peak b 150 mM KCl Peak c NaOH 1 M.

Fig. 15

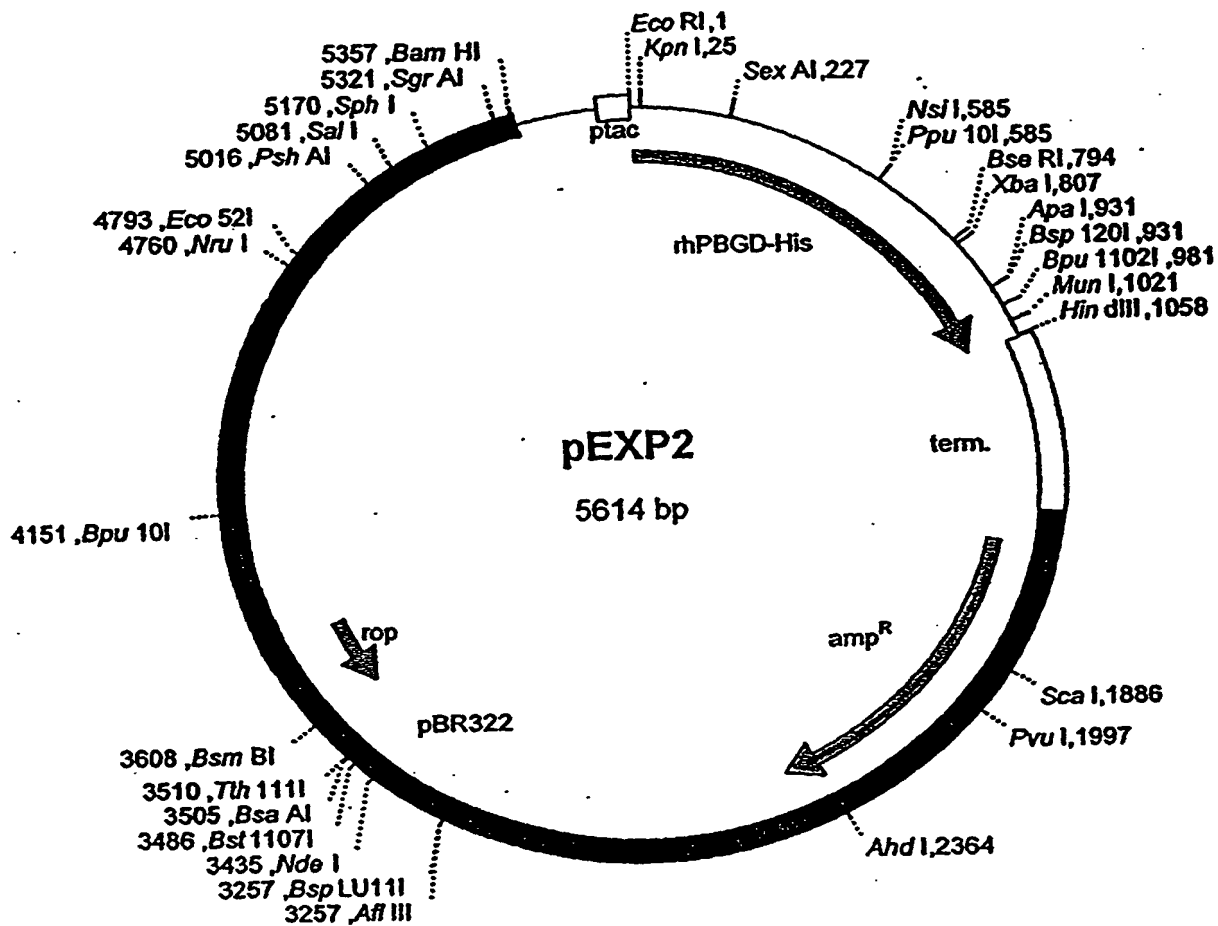
16/49



Chromatography on Butyl-Sepharose 4 FF. Peak a flow through the gel, Peak b water Peak c NaOH 1 M.

Fig. 16

17/49



PLASMID FEATURES:

Start	End	Name
7	1056	rhPBGD-His
1064	1490	terminator region
1582	2442	amp ^R
3820	3629	rop
5362	1490	pBR322
5544	1	ptac (promoter)

Fig. 17

18/49

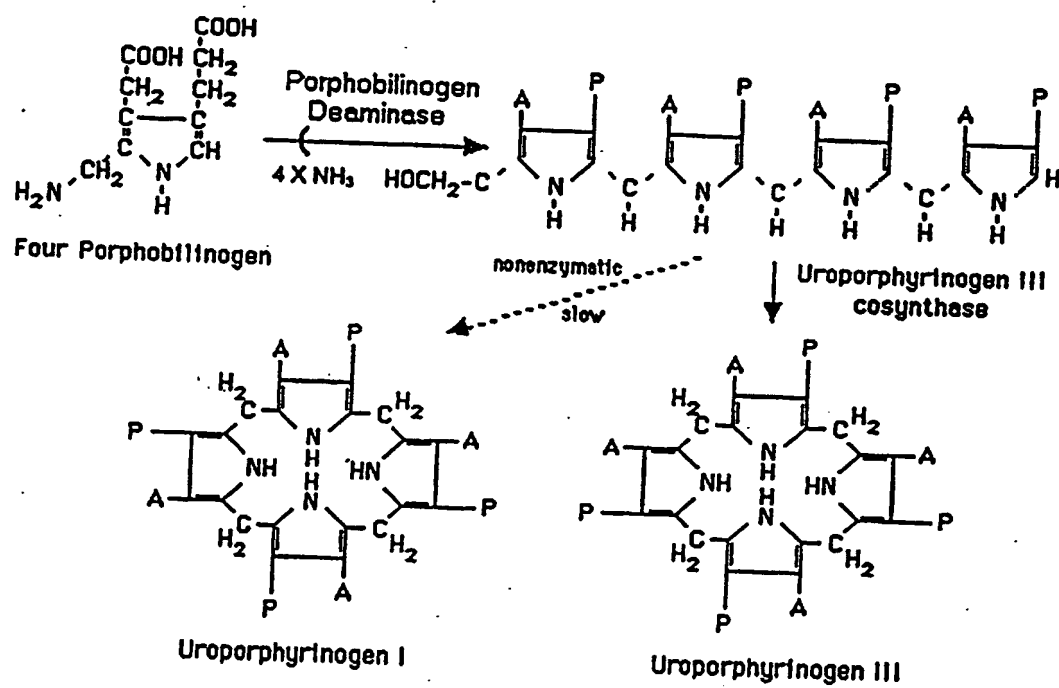


Fig. 18

19/49

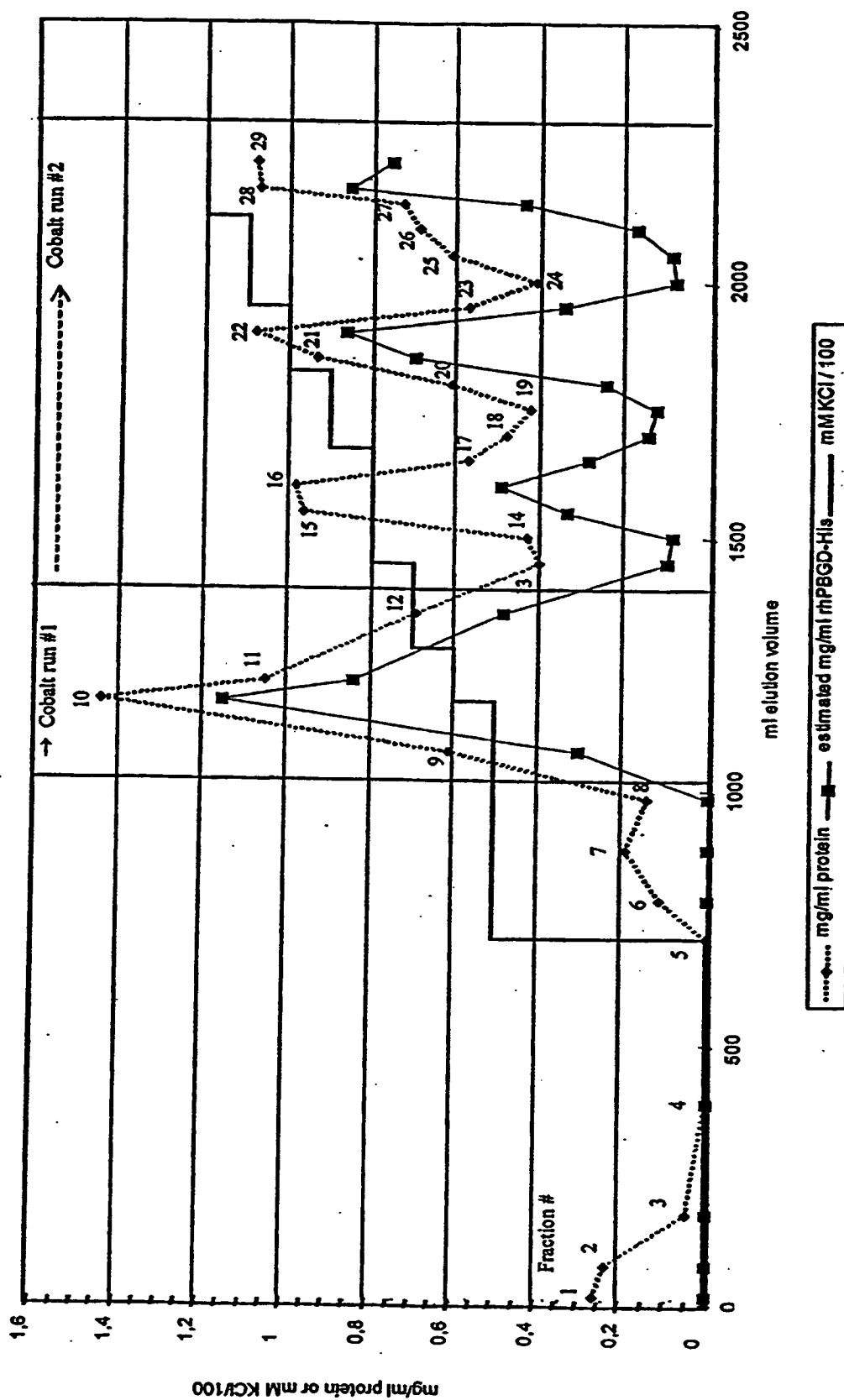
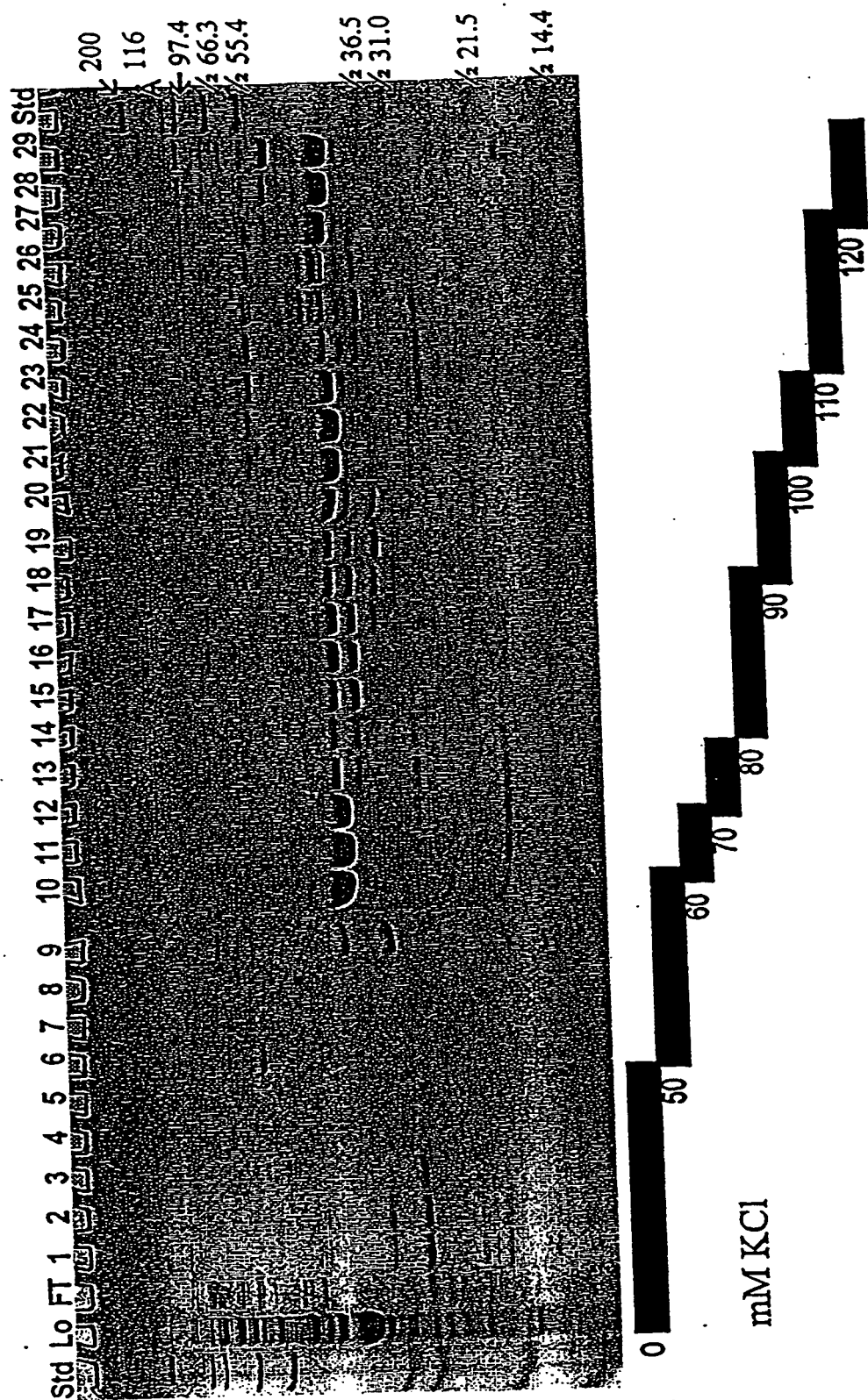


Fig. 19

20/49

DEAE Sepharose Column Fractions:



21/49

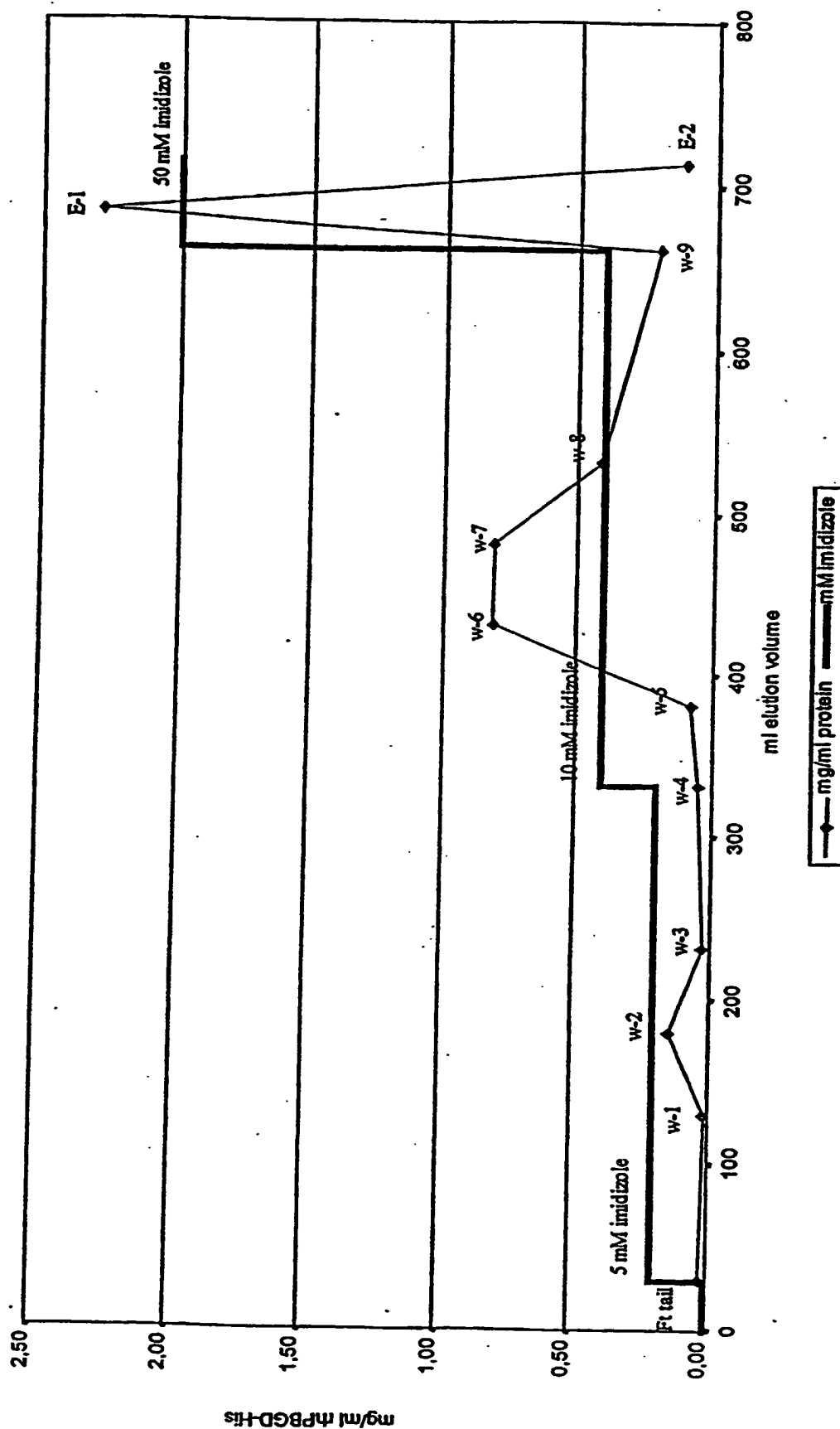


Fig. 21

22/49

1	Mark 12 molecular weight standards (Novex)
2	Cobalt load #1 (DEAE fractions 9 -> 12)
3	Flowthrough from cobalt load #1
4	50 mM imidazole eluate from cobalt run #1
5	Concatamerized nickel purified rhPBGD-His
6	Flowthrough from cobalt load #2
7	10 mM imidazole eluate from cobalt run #2
8	50 mM imidazole eluate from cobalt run #2

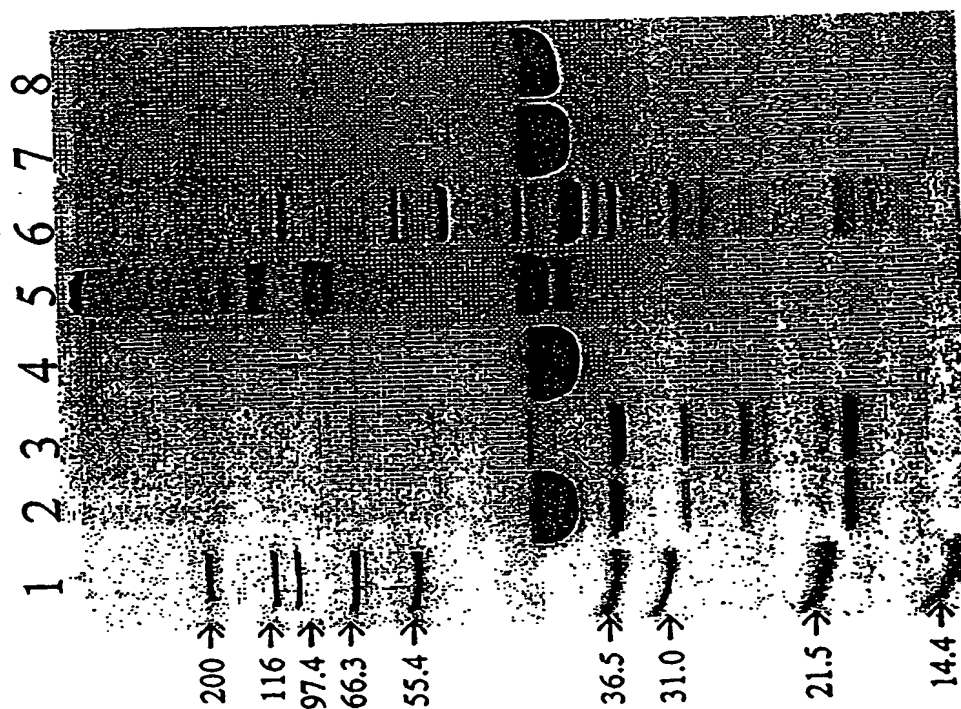


Fig. 22

23/49

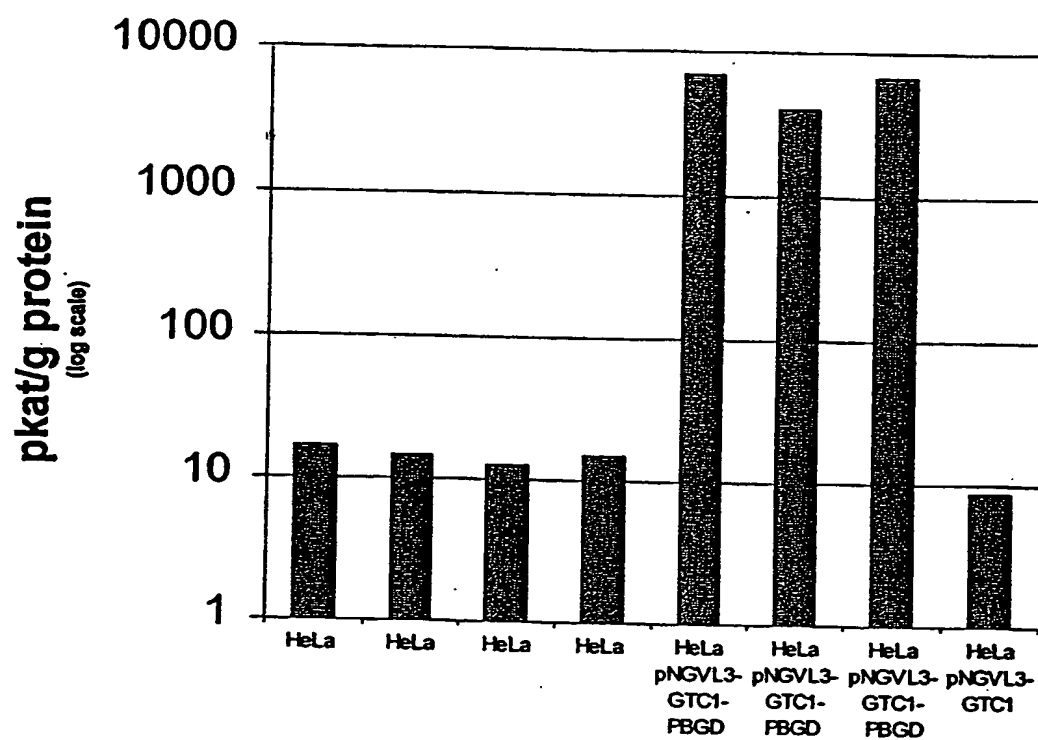


Fig 23: PBGD activity related to protein concentration in HeLa cells.

Fig. 23

24/49

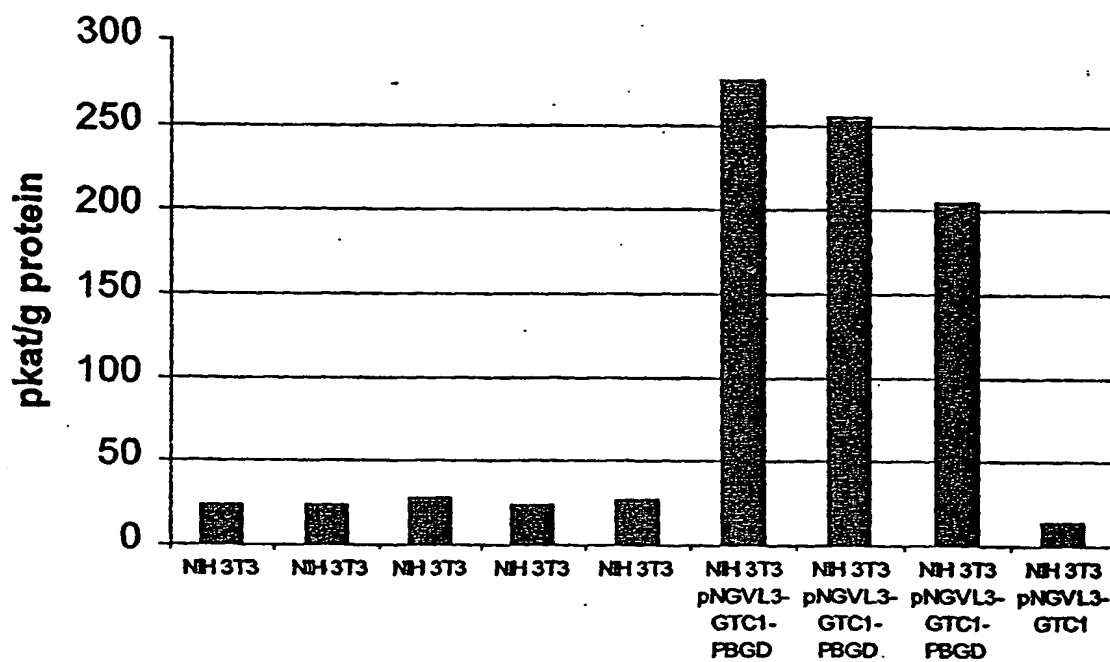


Figure 24: PBGD activity related to protein concentration in NIH 3T3 cells.

Fig. 24

25/49

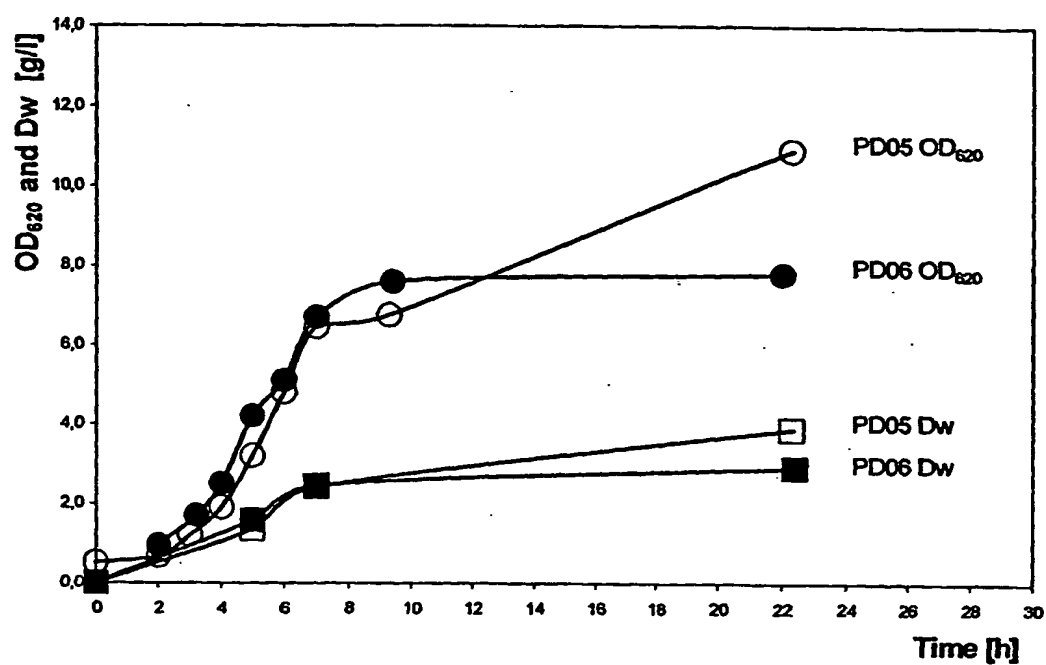


Fig 25. Comparison of fermentations PD05 and PD06 with strain PBGD-2.

Fig. 25

26/49

Fig 26. Comparison of fermentations PD09, PD11 and PD12

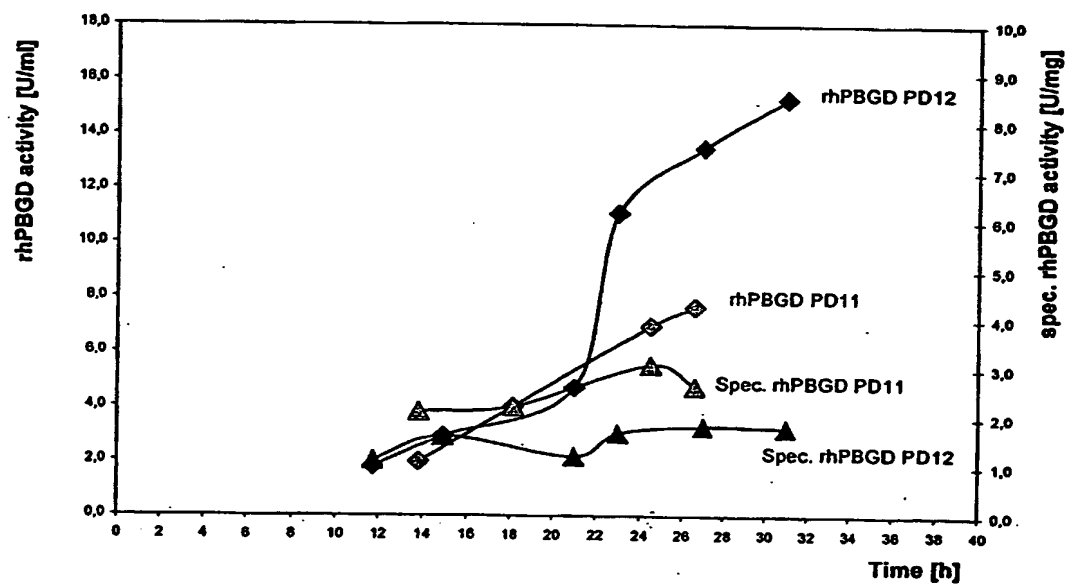


Fig. 26

27/49

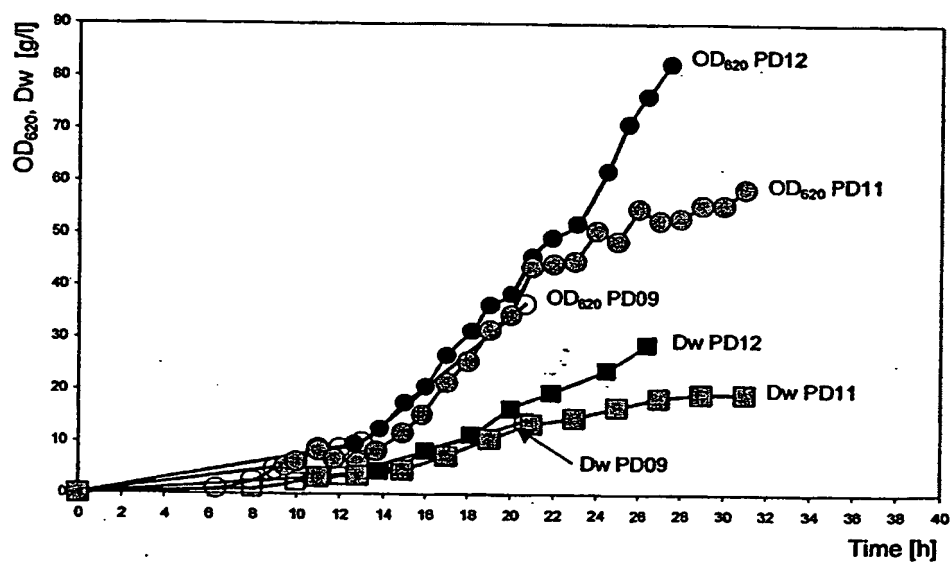


Fig 27: Comparison of fermentations PD09, PD11 and PD12 with strain PBGD-1.

Fig. 27

28/49

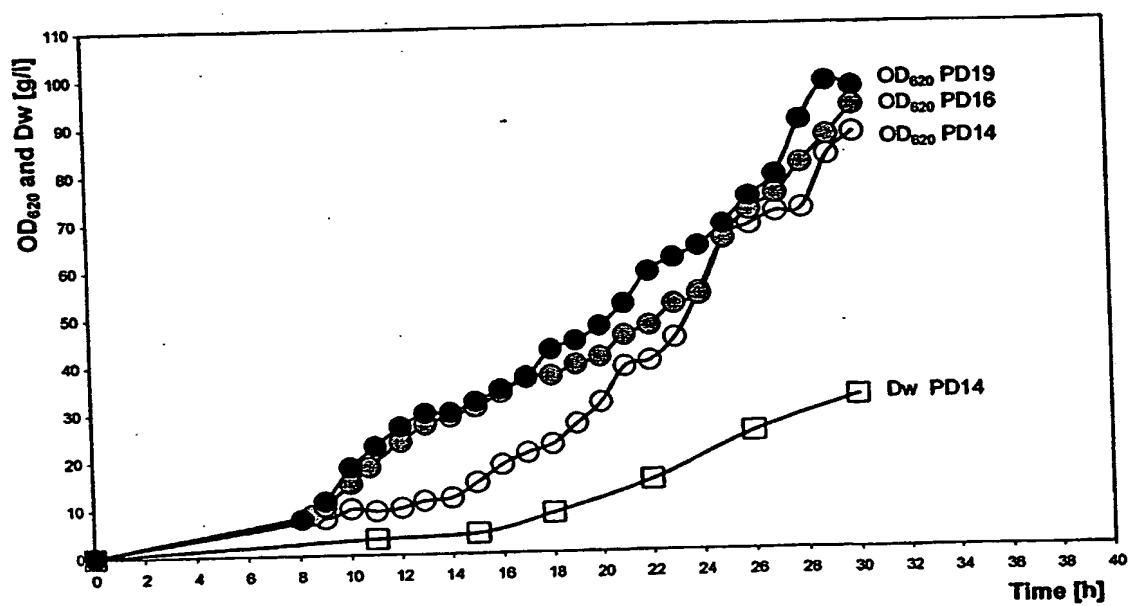


Fig 28. Comparison of fermentations PD14, PD16 and PD19 with strain PBGD-2.

Fig. 28

29/49

Fig 29. Comparison of fermentations PD14, PD16 and PD19 with strain PBGD-2.

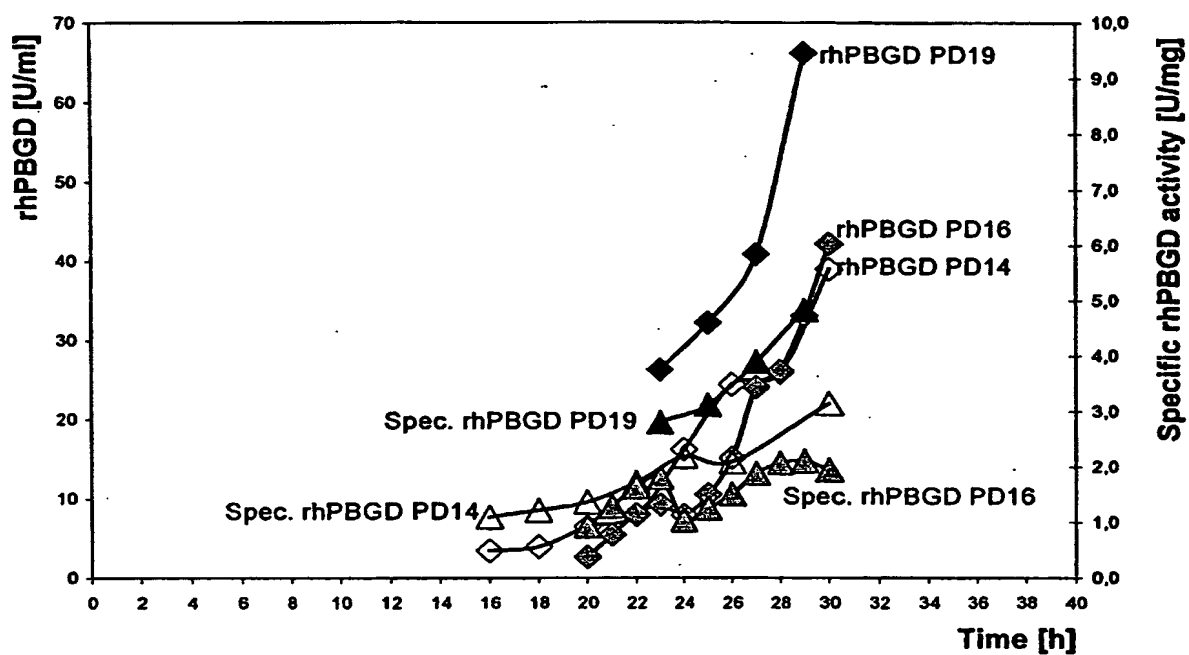


Fig. 29

30/49

Fig 30. Comparison of fermentations PD19, PD21 and PD22 with strain PBGD-2.

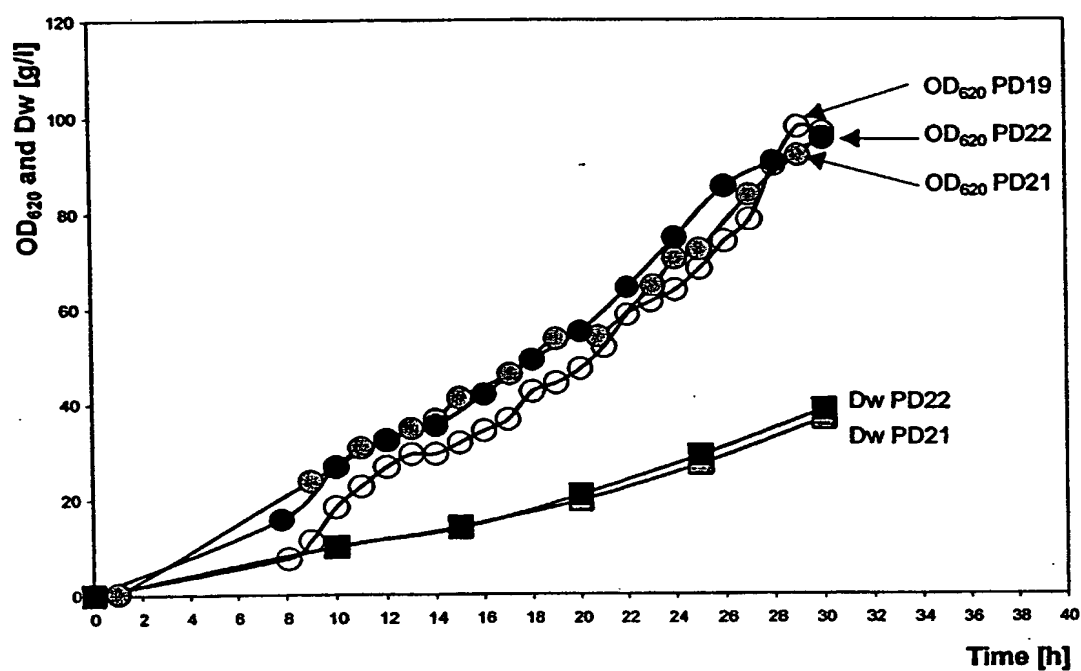


Fig. 30

31/49

Fig 31. Comparison of fermentations PD19, PD21 and PD22 with strain PBGD-2.

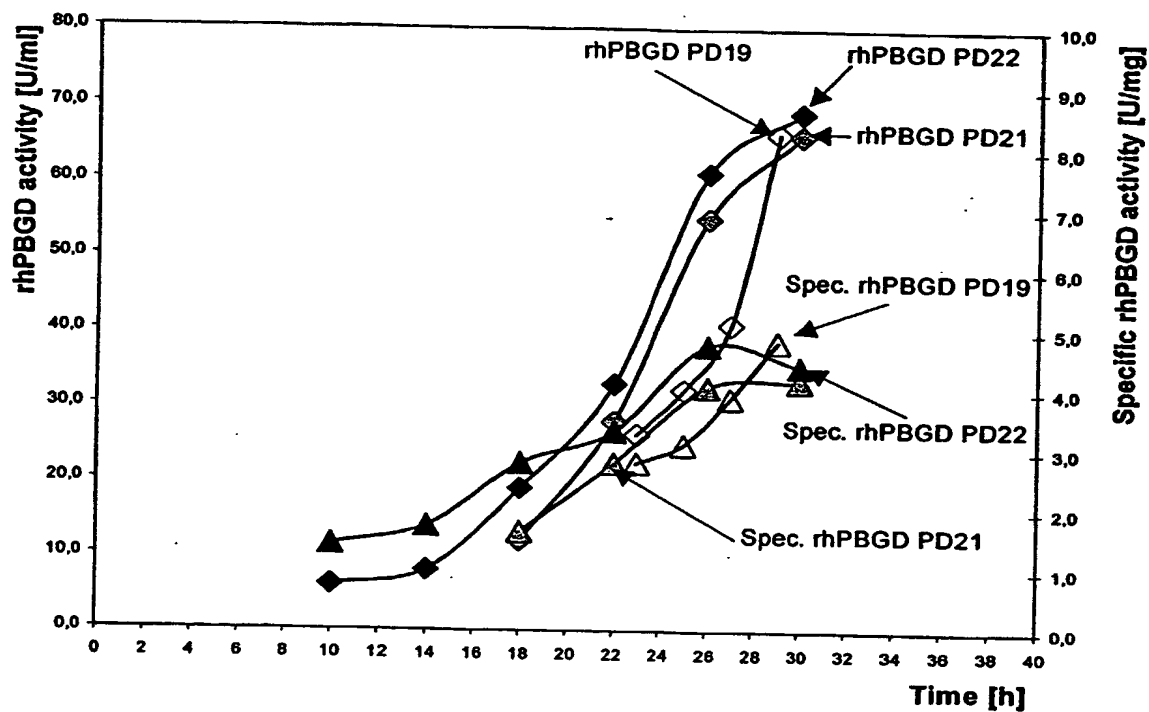


Fig. 31

32/49

Fig 32. Comparison of fermentations PD19, PD1501 and PD1502.

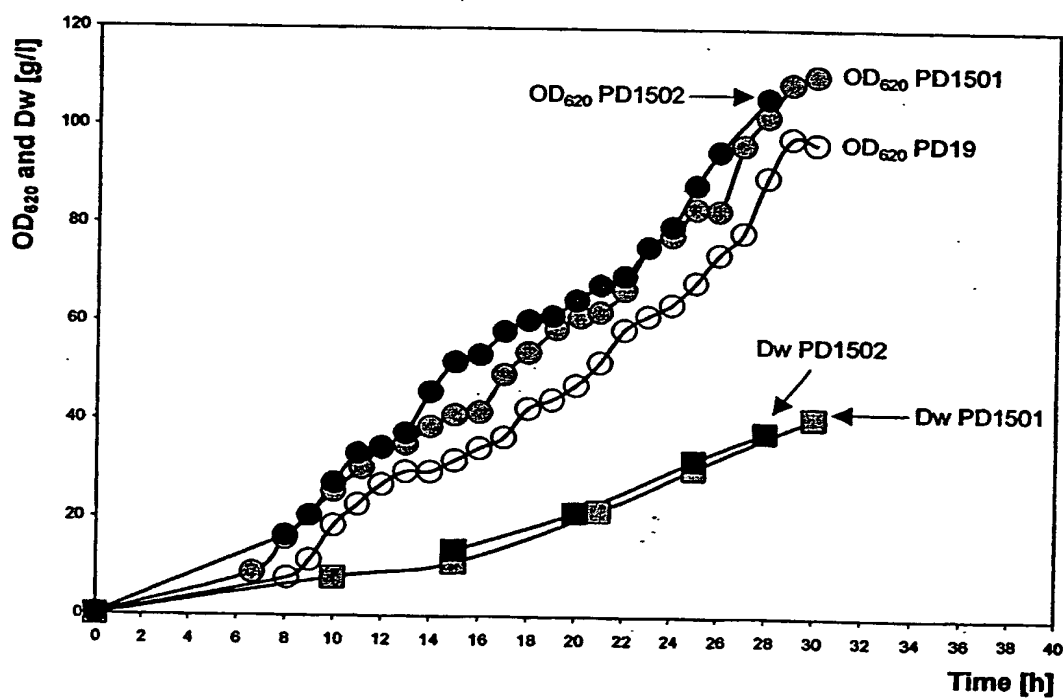


Fig. 32

33/49

Fig 33. Comparison of fermentations PD19, PD1501 and PD1502 with strain PBGD-2.

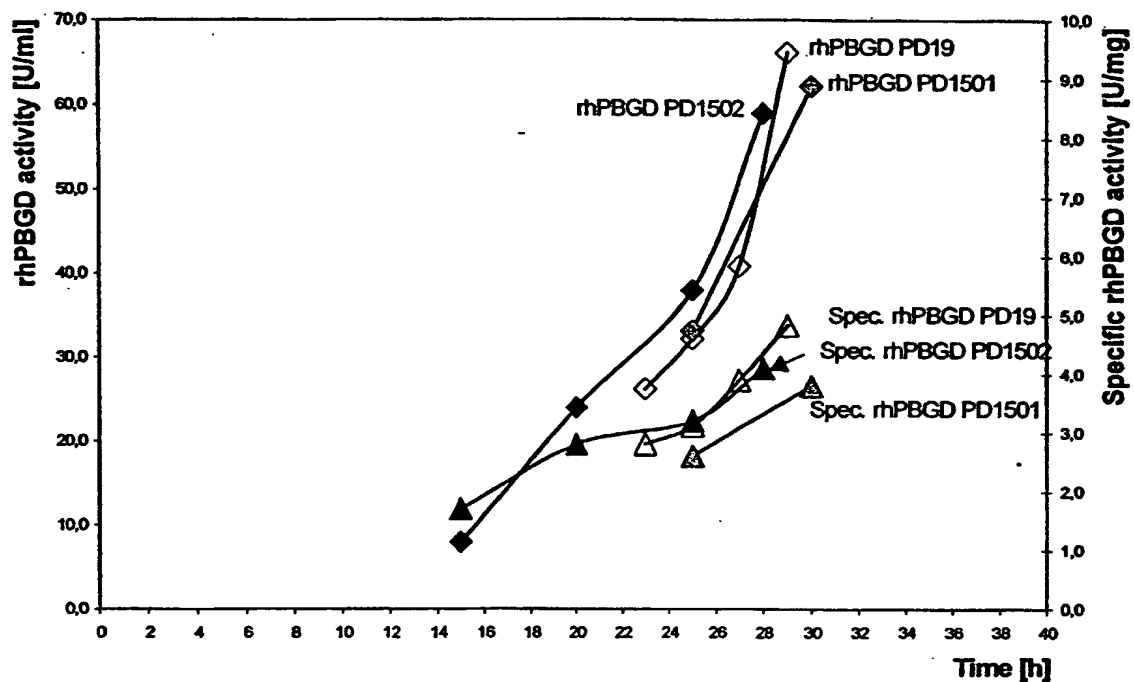
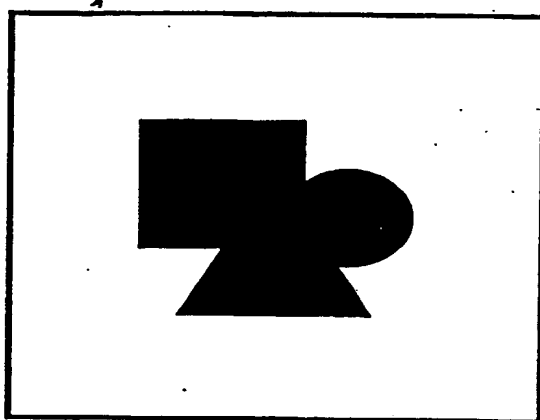


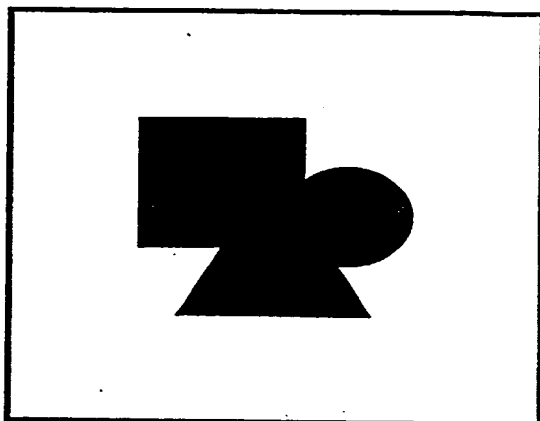
Fig. 33

34/49

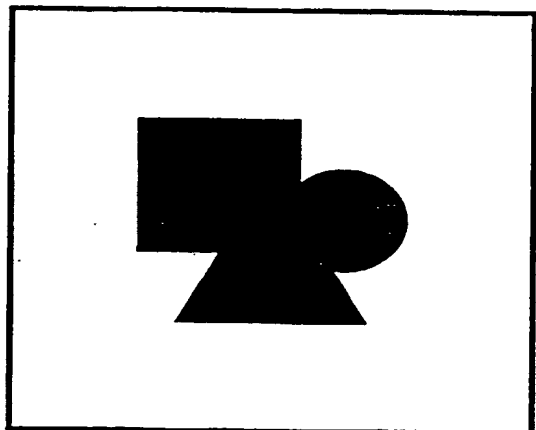
Summary of Fermentation and down stream process visualized by SDS-Page.
Comparison between different samples.



Well	Samples from PD22
1	Mark 12 MW st
2	Extract
3	Homogenate
4	Broth 30 h
5	Broth 26 h
6	Broth 22 h
7	Broth 18 h
8	Broth 14 h
9	Broth 10 h
10	rhPBGD-His



	Samples from PD1501
1	Mark 12 MW st
2	Extract
3	Extract
4	Extract
5	Broth 30 h
6	Broth 30 h
7	Broth 25 h
8	Broth 20 h
9	Broth 15 h
10	rhPBGD-His



	Samples from PD1502
1	Mark 12 MW st
2	Broth 15 h
3	Broth 20
4	Broth 25 h
5	Broth 28 h
6	Homogenate
7	Extract
8	Extract
9	Extract
10	rhPBGD-His

Fig. 34

35/49

Fig 35. Stability studies: Single use aliquots of extract were routinely taken out of the freezer (-20°C) and the rhPBGD-activity was measured and plotted over time.

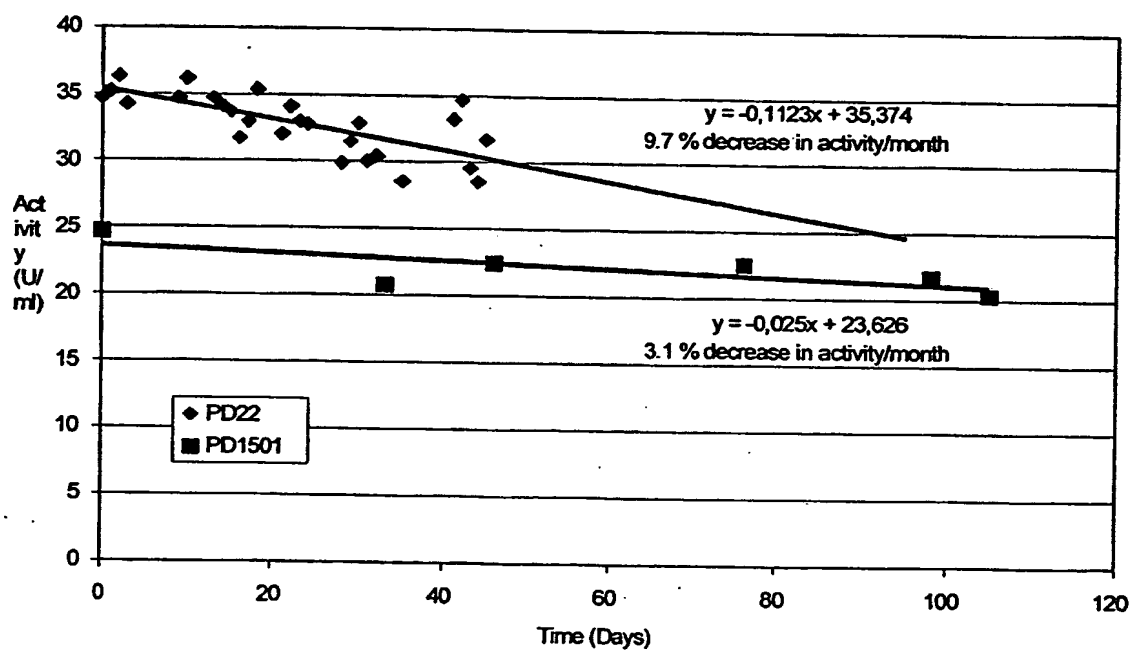
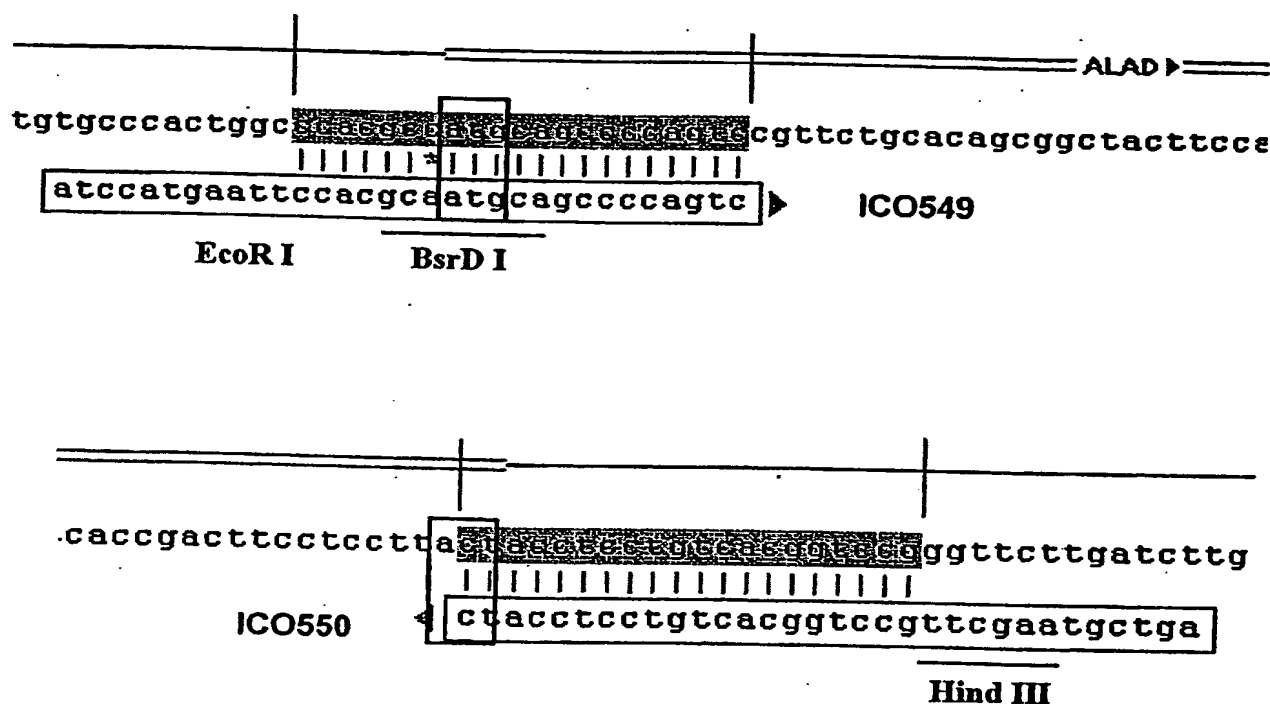


Fig. 35

36/49

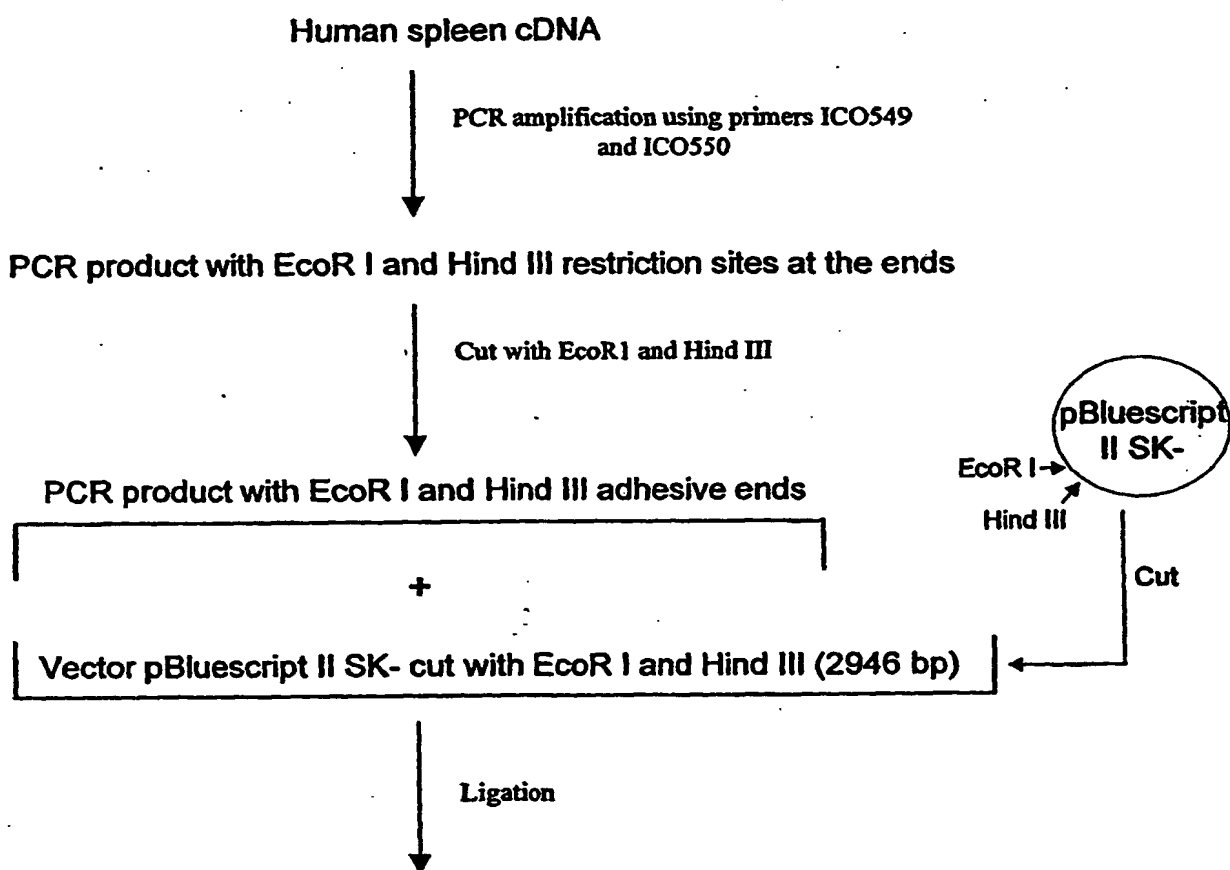
Description of the oligos used for PCR amplification



The two oligos ICO549 and ICO550 are shown below the ALAD sequence. The highlighted regions in the ALAD sequence represent the regions of homology with the oligos. The asterisk denotes the C to A change introduced to generate a *BsrD* I site for ease of manipulation of the 5' end of the cloned molecule. The initiation and termination triplets in the ALAD sequence are boxed. Both the oligos have 5' sequences extending beyond the regions of homology to incorporate restriction sites for cloning of the amplified DNA, viz. *EcoR* I for ICO549 and *Hind* III for ICO550.

Fig. 36

37/49

Strategy for PCR cloning of ALAD**Fig. 37**

38/49

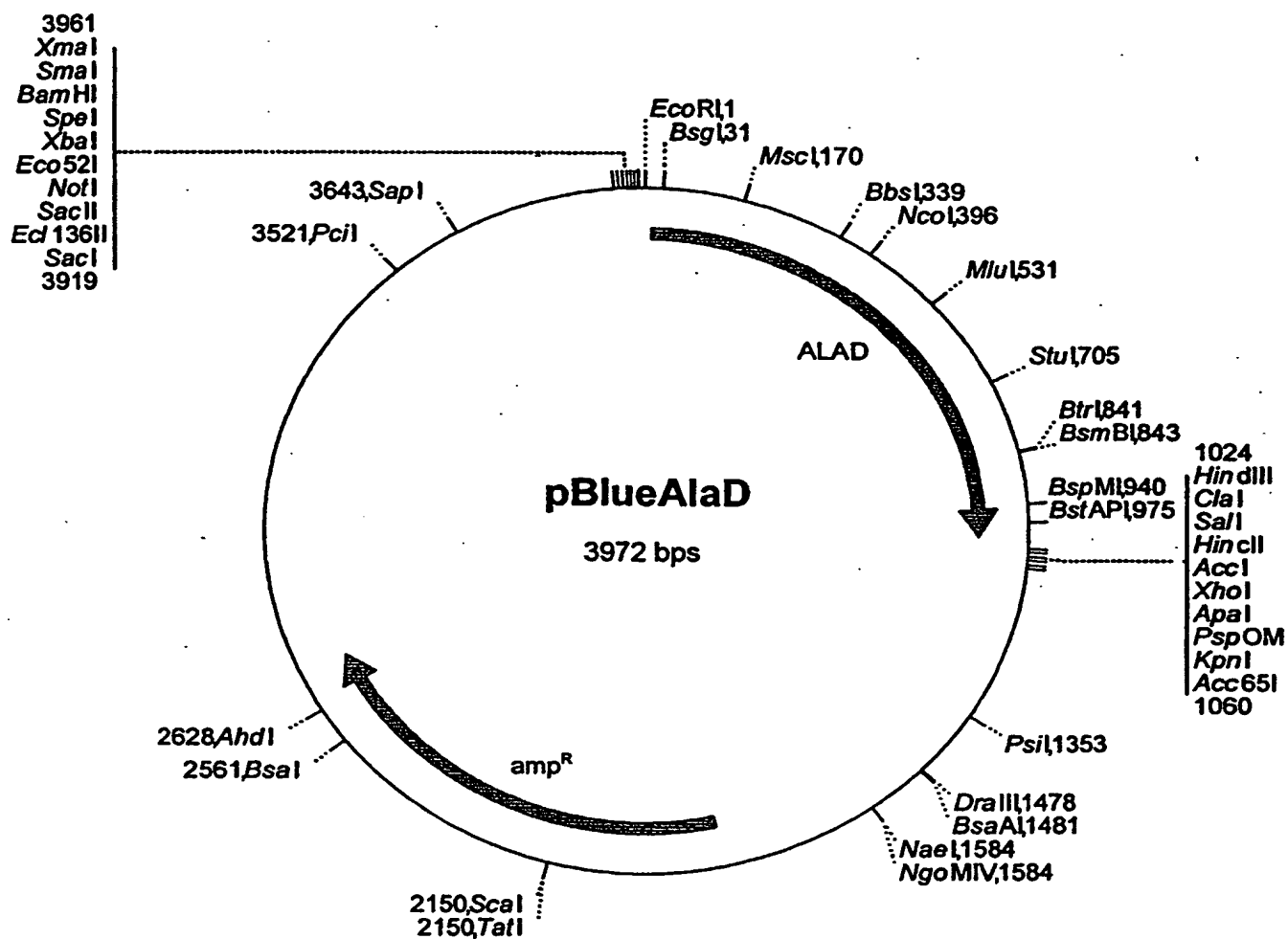


Fig. 38

39/49

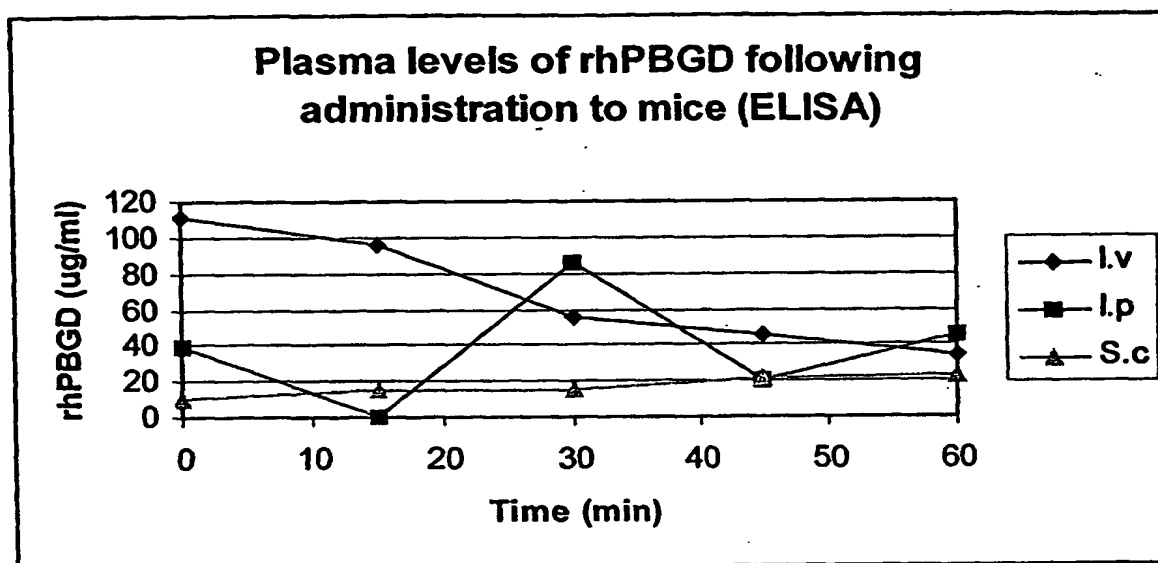


Figure 38: Plasma levels of rhPBGD following administration to mice.

Fig. 39

SUBSTITUTE SHEET (RULE 26)

40/49

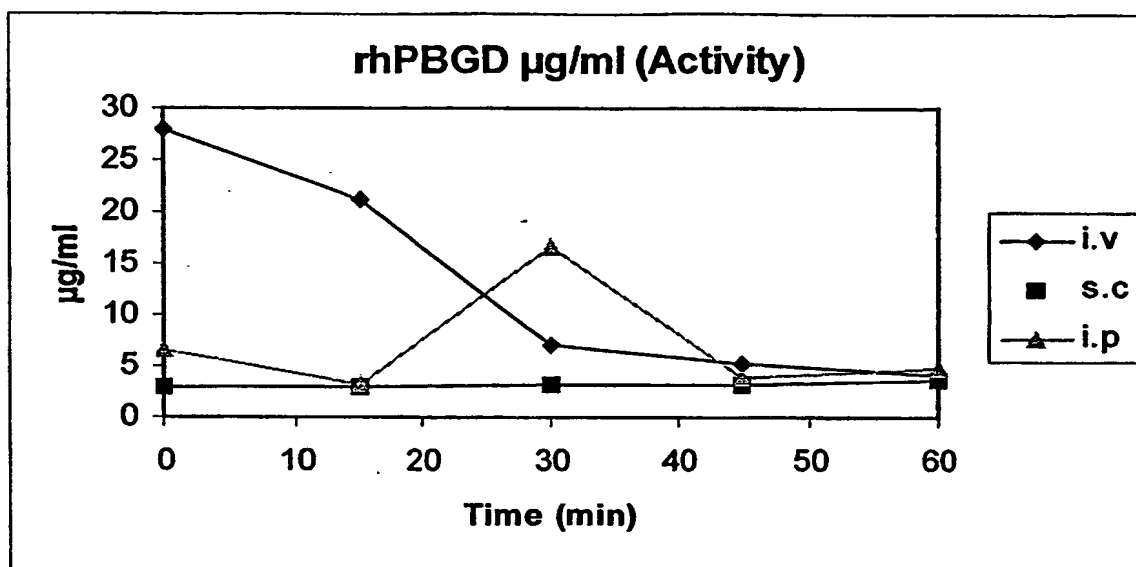


Figure 39: PBGD enzymatic activity in plasma following rhPBGD administration to mice

Fig. 40

41/49

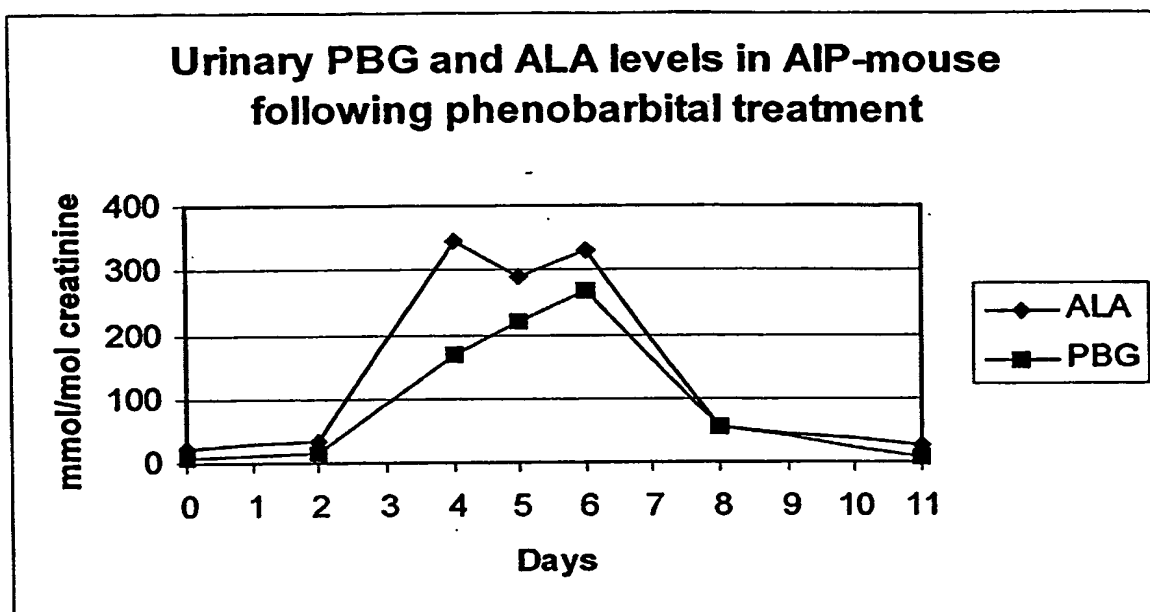


Figure 40: Urinary content of PBG and ALA in AIP-mouse treated with phenobarbital.

Fig. 41

42/49

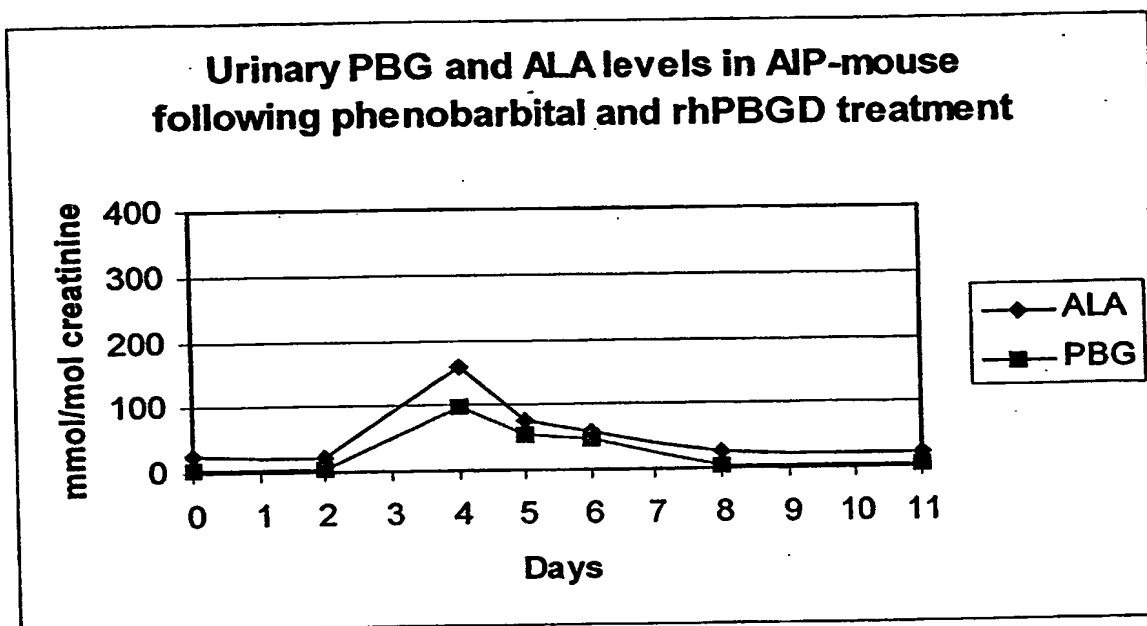


Figure 41: Urinary content of PBG and ALA in AIP-mouse treated with phenobarbital and rhPBGD. Mice were treated with an increasing dose of phenobarbital for 4 days (day 0-4,

Fig. 42

43/49

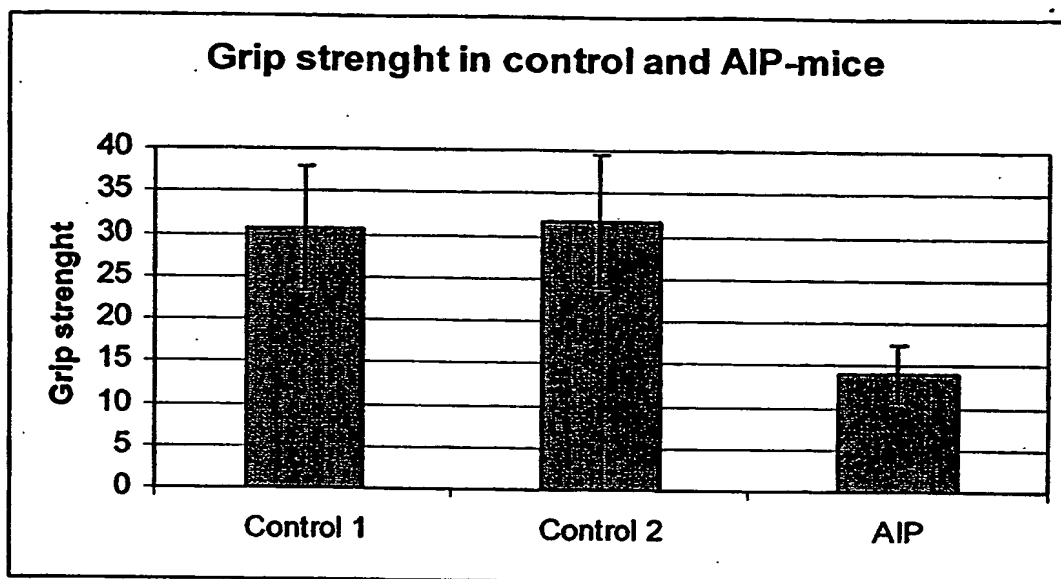


Figure 42: Grip strength analysis in control and AIP-mice. Grip strength were determined using a grip strength meter (Ugo Basile) in heterozygous control animals (control 1, n=5), in wild type controls (control 2, n=5) and in AIP-transgenic mice (AIP, n=5).

Fig. 43

44/49

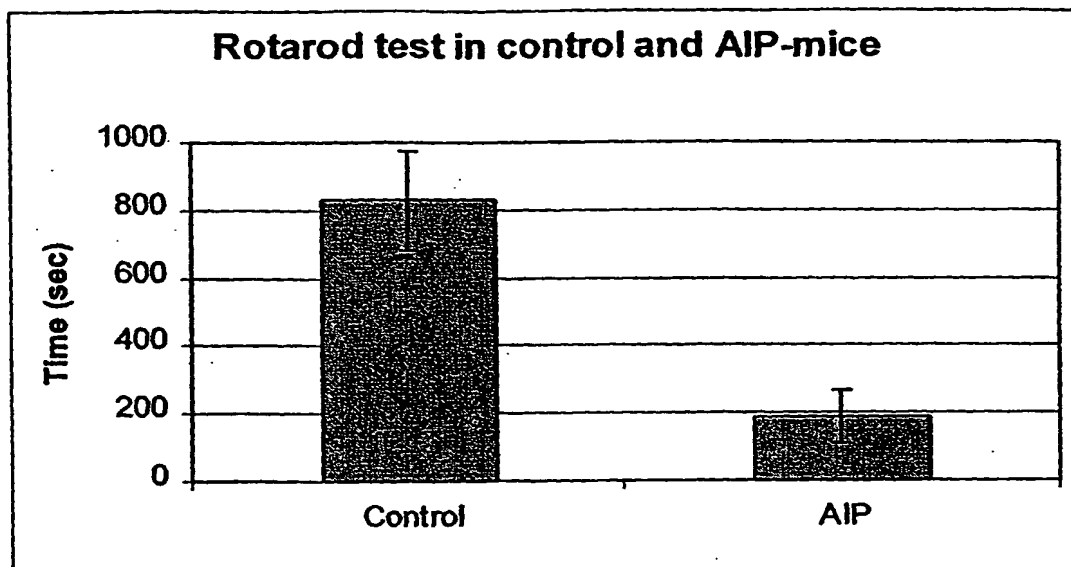


Figure 4 Rotarod analysis in control and AIP-mice. The rotarod analysis were determined using a rotarod treadmill (Ugo Basile) in wild type controls (control, n=5) and in AIP-transgenic mice (AIP, n=7).

Fig. 44

45/49

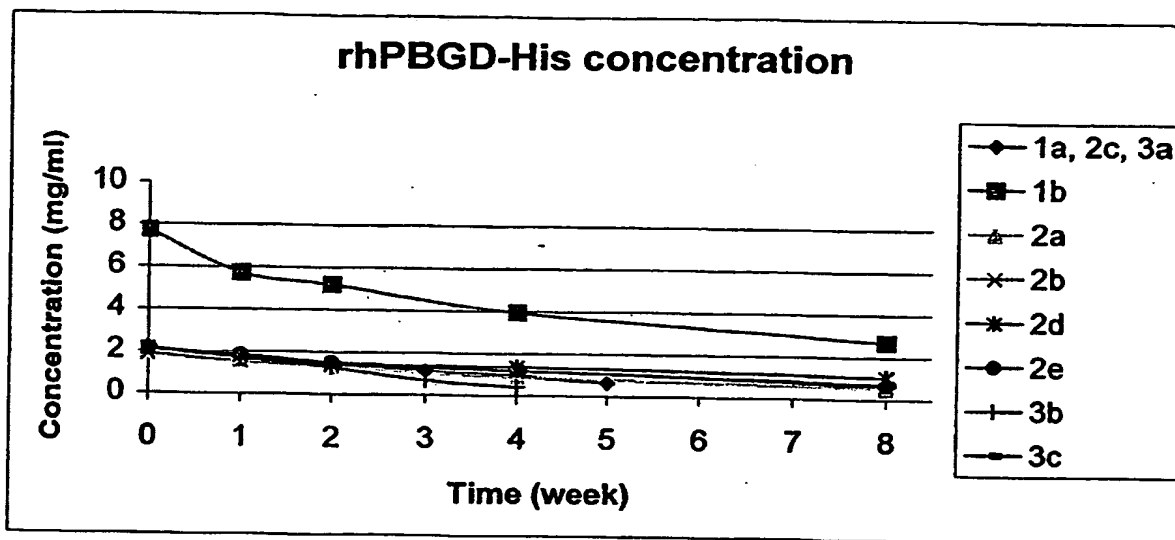


Figure 44. Enzyme concentration over 8 weeks at 40°C measured by HPLC. A decrease from 2 mg/ml to 0,5 mg/ml and 8 mg/ml to 2,5 was detected.

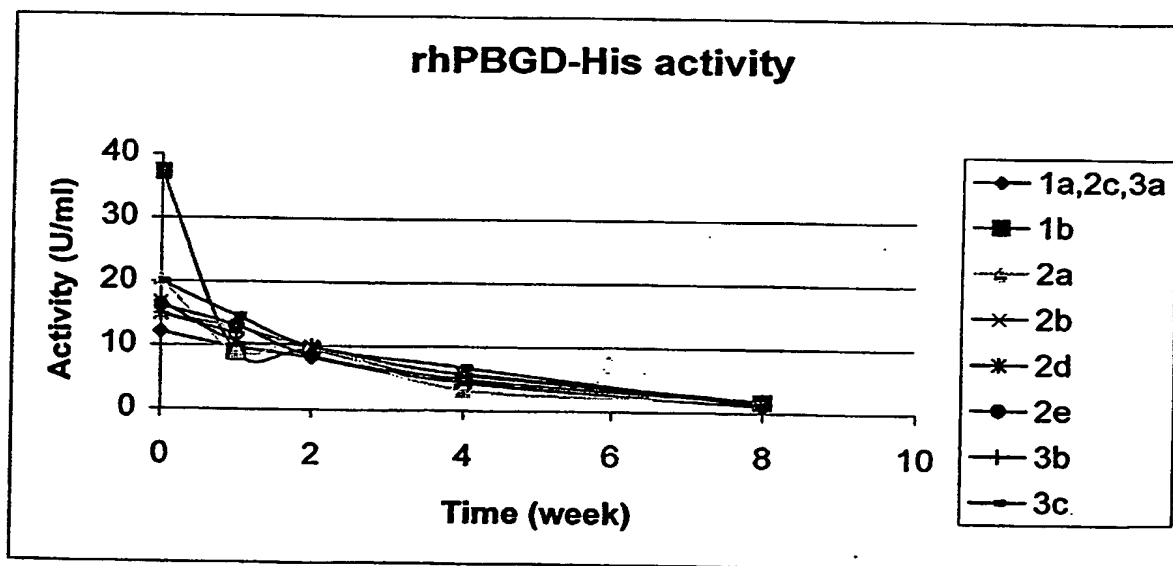


Figure 45. The enzyme activity measured over 8 weeks at 40°C. A significant decrease over the first week was seen for the high concentration sample, 1b. After two weeks the decrease rate was the same for all samples.

Fig. 45

46/49

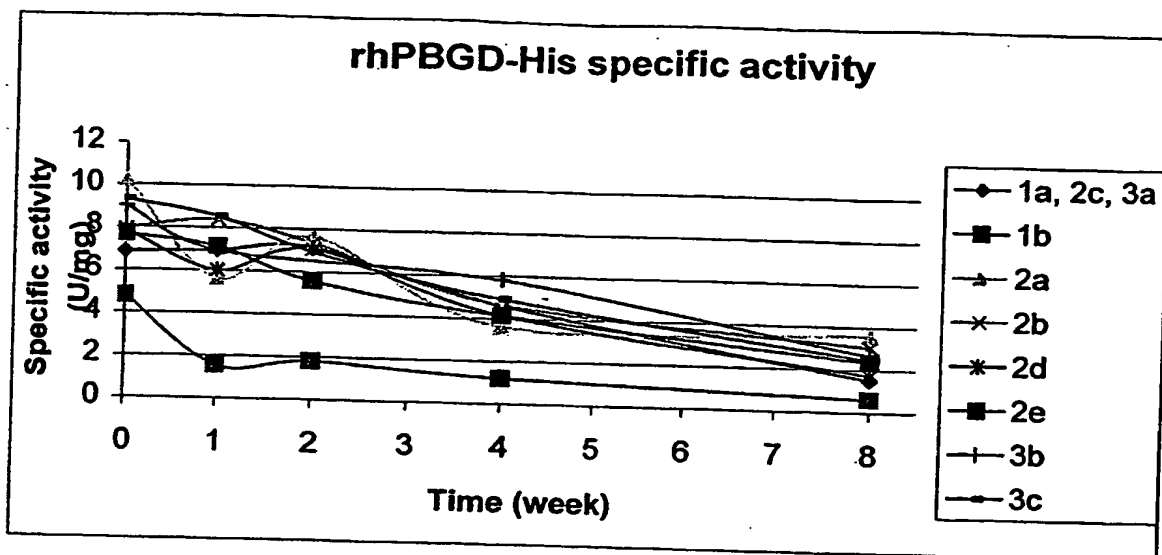


Figure 46. Enzyme specific activity measured during 8 weeks at 40°C. The activity was measured using the enzyme activity assay and the protein concentration was measured using HPLC.

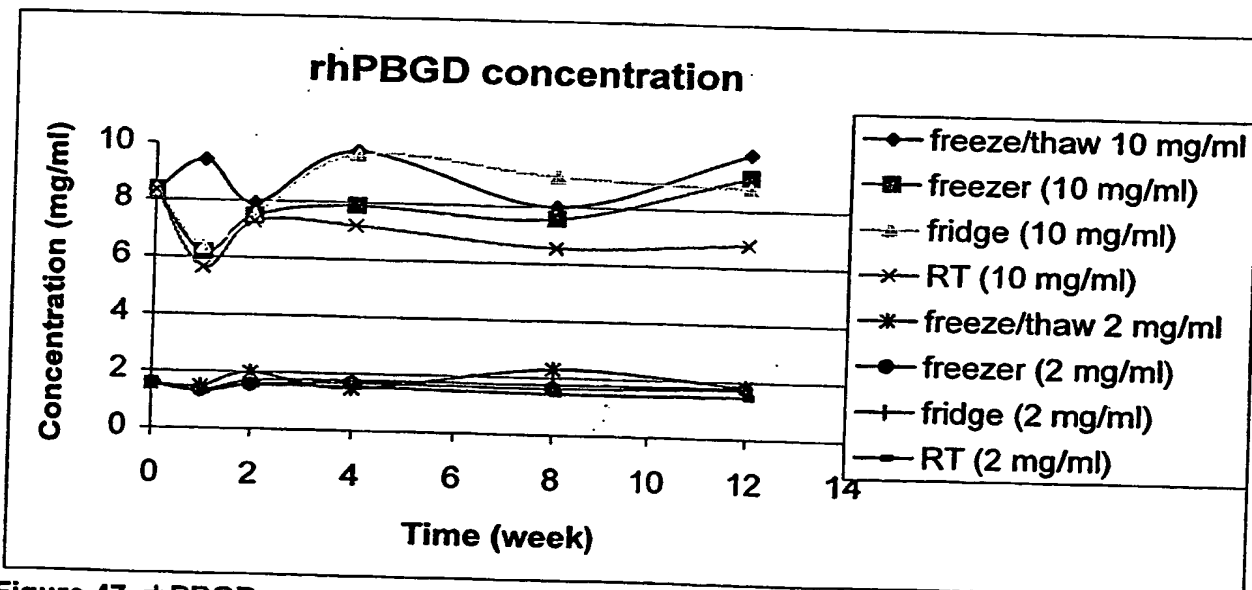


Figure 47. rhPBGD concentration over 12 weeks at -20°C (freezer), 5°C (fridge), 25°C (RT) and freeze/thawed at each sampling. The measurement was performed using HPLC.

Fig. 46

47/49

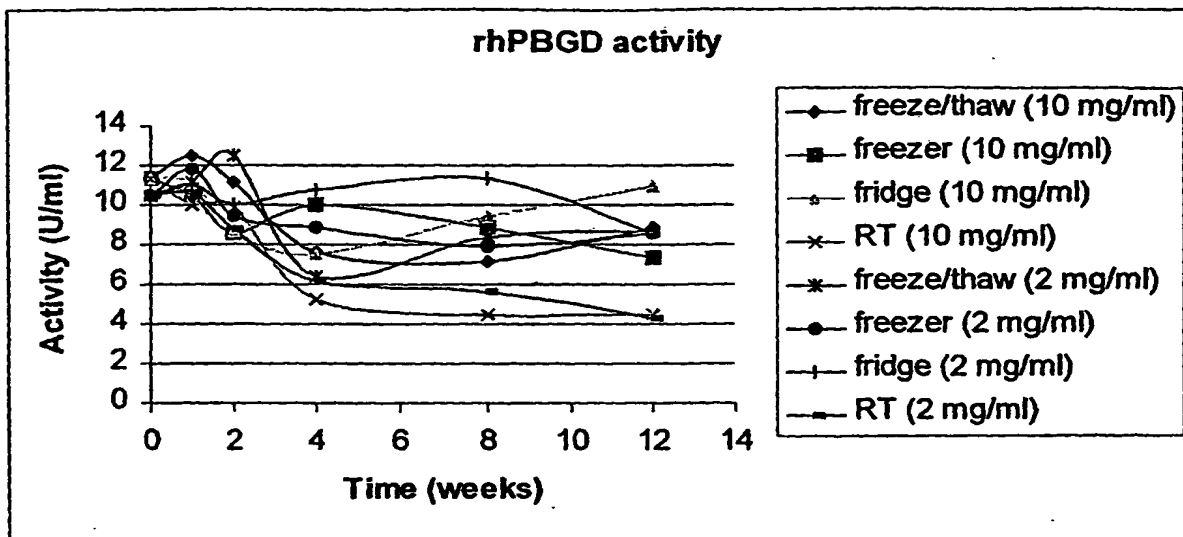


Figure 48. rhPBGD activity over 12 weeks at -20°C (freezer), 5°C (fridge), 25°C (RT) and freeze/thawed at each sampling.

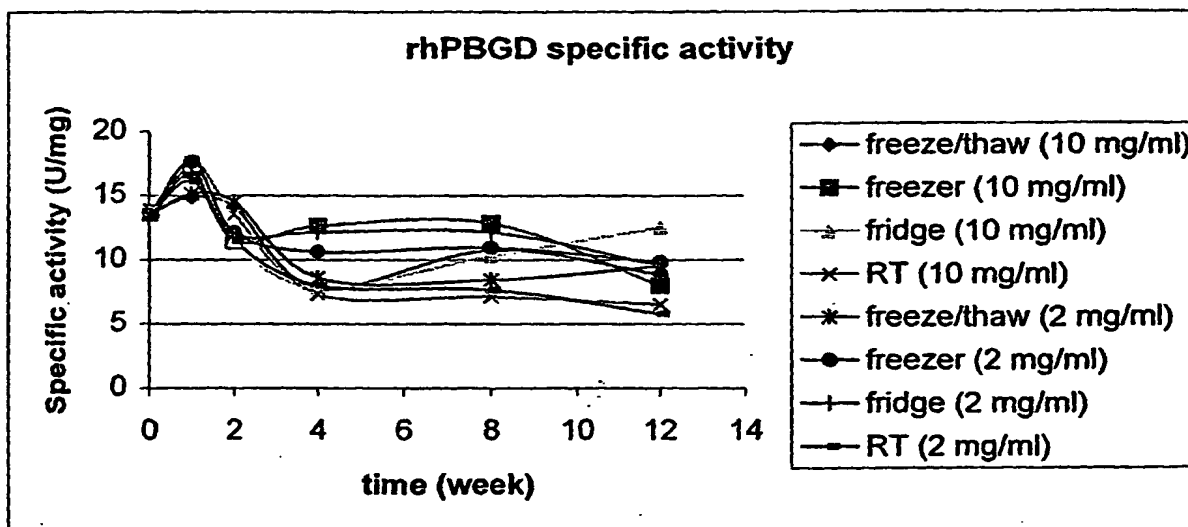


Figure 49. rhPBGD specific activity over 12 weeks at -20°C (freezer), 5°C (fridge), 25°C (RT) and freeze/thawed at each sampling. Measurements were performed using enzyme activity assay and HPLC.

Fig. 47

48/49

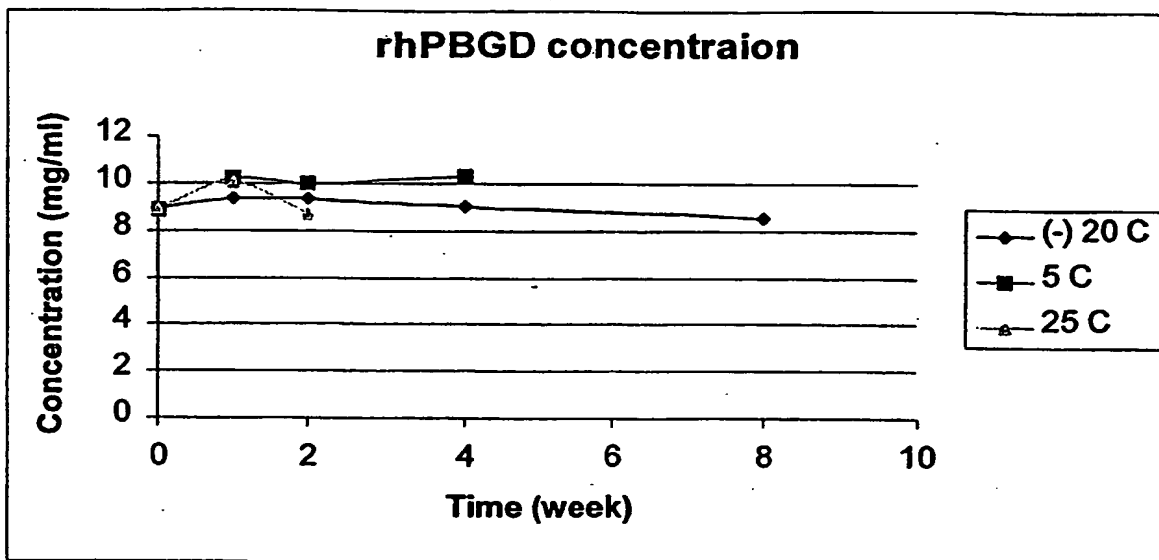


Figure 50. rhPBGD concentration measured over 8 weeks using BCA.

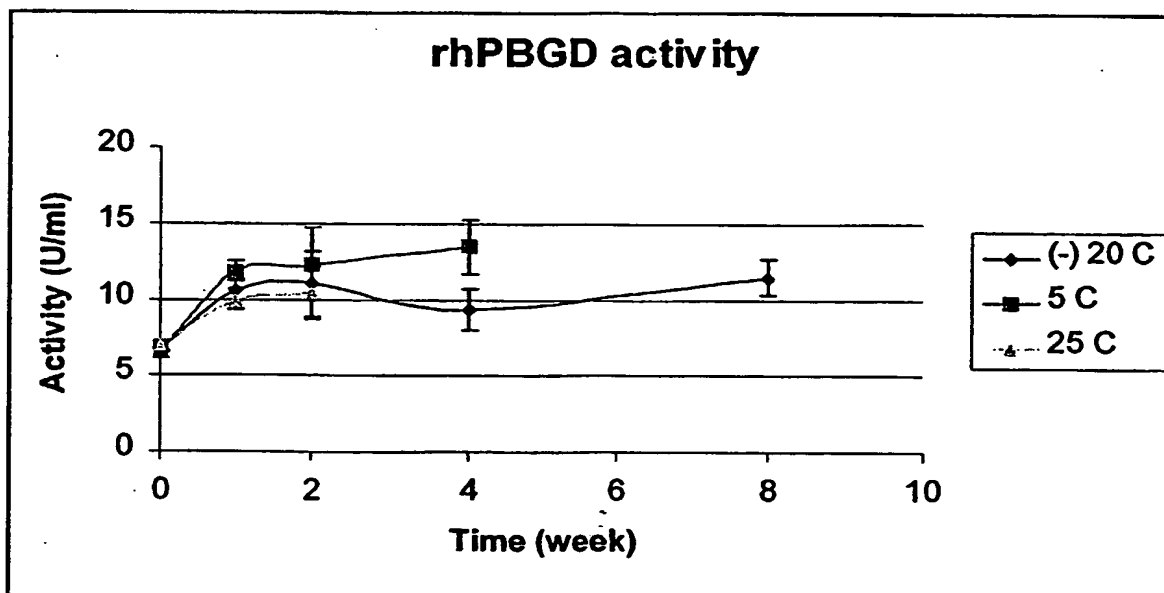


Figure 51. The rhPBGD activity measured over 8 weeks. The stability study has been performed under nitrogen at $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$, $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$ and at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$.

Fig. 48

49/49

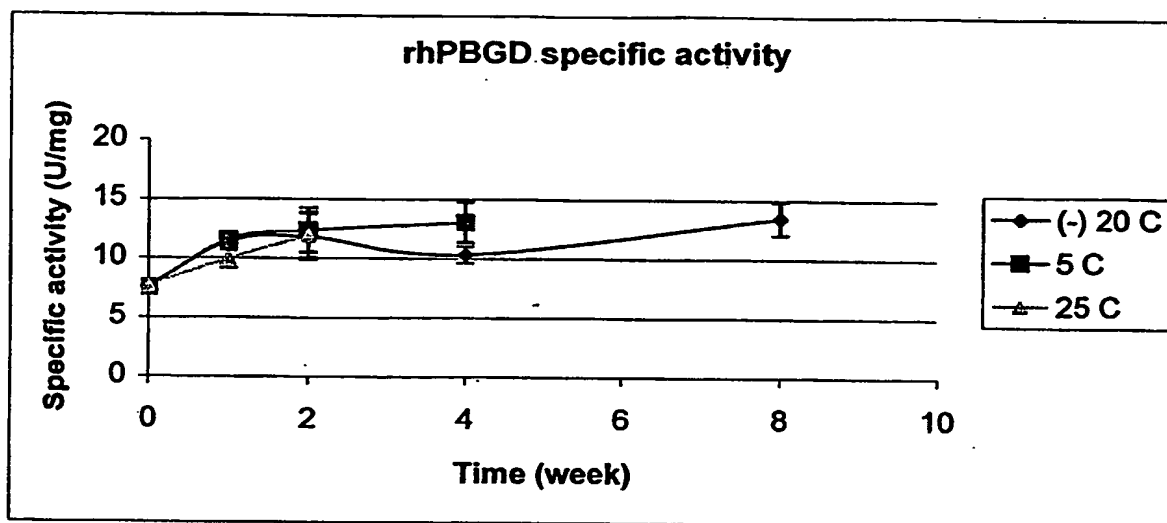


Figure 52. The specific rhPBGD activity measured using the enzyme activity assay and BCA protein concentration assay. The stability study has been performed under nitrogen at $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$, $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$ and at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$.

Fig. 49

SEQUENCE LISTING

<110> HEMEBIOTECH A/S

<120> PRODUCTION OF rhPBGD AND NEW THERAPEUTIC
METHODS FOR TREATING PATIENTS WITH ACUTE INTERMIT-TENT
PORPHYRIA (AIP) AND OTHER PORPHYRIC DISEASES

<130> 23725PC1

<160> 22

<170> FastSEQ for Windows Version 3.0

<210> 1

<211> 5446

<212> DNA

<213> Homo sapiens

<400> 1

gaattctaac	ataagttaag	gaggaaaaaa	aaatgagagt	tattcgtgtc	ggtacccgca	60
agagccagct	tgctcgcata	cagacggaca	gtgtgggtggc	aacattgaaa	gcctcgtacc	120
ctggcctgca	gtttgaaatc	attgctatgt	ccaccacagg	ggacaagatt	cttgatactg	180
cactctctaa	gattggagag	aaaagcctgt	ttaccaagga	gcttgaacat	gccctggaga	240
agaatgaagt	ggacctgggt	gttcactcct	tgaaggacct	gccactgtg	cttcctcctg	300
gcttcaccat	cggagccatc	tgcaagcggg	aaaaccctca	tgatgctgtt	gtctttcacc	360
caaaatttgt	tggaagacc	ctagaaaccc	tgccagagaa	gagtgtgggtg	ggaaccagct	420
ccctgcgaag	agcagcccag	ctgcagagaa	agttcccgcga	tctggagtgc	aggagtattc	480
ggggaaacct	caacacccgg	cttcgggaagc	tggacgagca	gcaggagtgc	agtgccatca	540
tcttggaac	agctggcctg	cagcgcattg	gctggcaca	ccgggttggg	cagatcctgc	600
accctgagga	atgcatgtat	gctgtggggc	agggggcctt	gggcgtggaa	gtgcgagcca	660
aggaccagga	catcttggat	ctggtgggtg	tgctgcacga	tcctcgagact	ctgcttcgct	720
gcatcgctga	aagggccttc	ctgaggcacc	tggaggagg	ctgcagtgtg	ccagtagccg	780
tgcatacagc	tatgaaggat	gggcaactgt	acctgactgg	aggagtctgg	agtctagacg	840
gctcagatag	catacaagag	accatgcagg	ctaccatcca	tgtccctgcc	cagcatgaag	900
atggccctga	ggatgaccca	cagttggtag	gcatcactgc	tcgtaacatt	ccacgagggc	960
cccagttggc	tgcccgaac	ttgggcatca	gcctggccaa	cttgttgctg	agcaaaggag	1020
ccaaaaacat	cctggatgtt	gcacggcaat	tgaacgatgc	ccattaataa	gcttggctgt	1080
tttgccggat	gagagaagat	tttcagcctg	atacagatta	aatcagaacg	cagaagcggg	1140
ctgataaaac	agaatttgcc	tggcggcagt	agcgcgggtg	tcctccctga	ccccatgccg	1200
aactcagaag	tgaaacgccg	tagcggccgat	ggtagtgtgg	ggctctcccca	tgcgagagta	1260
gggaactgcc	aggcatcaaa	taaaacgaaa	ggctcagtcg	aaagactggg	cctttcgttt	1320
tatctgttgt	ttgtcgggtga	acgctctcct	gagtaggaca	aatccgcccgg	gagcggattt	1380
gaacgttgcg	aagcaacggc	ccggagggtg	gcgggcagga	cgcccgccat	aaactgccag	1440
gcatcaaatt	aagcagaagg	ccatcctgac	ggatggcctt	tttgcgtttc	tacaaactct	1500
tttgtttatt	tttctaaata	cattcaaata	tgtatccgct	catgagacaa	taaccctgat	1560
aaatgcttca	ataatattga	aaaagggaaga	gtatgagtat	tcaacatttc	cgtgtcgccc	1620
ttattccctt	ttttgcggca	ttttgccttc	ctgtttttgc	tcacccagaa	acgctgggtga	1680
aagtaaaaga	tgctgaagat	cagttgggtg	cacgagtggg	ttacatcgaa	ctggatctca	1740
acagcggtaa	gatccttgag	agttttcgcc	ccgaagaacg	ttttccaatg	atgagcactt	1800
ttaaagttct	gctatgtggc	gcggtattat	cccgtgttga	cgccggggcaa	gagcaactcg	1860
gtcgccgcac	acactattct	cagaatgact	tggttgagta	ctcaccagtc	acagaaaagc	1920
atcttacgga	tgcatgaca	gtaagagaat	tatgcagtgc	tgccataacc	atgagtata	1980
acactgcggc	caacttactt	ctgacaacga	tcggaggacc	gaaggagcta	accgcttttt	2040
tgacaacat	gggggatcat	gtaactcgcc	ttgatcgttg	ggaaccggag	ctgaatgag	2100
ccataccaaa	cggcagcgt	gacaccacga	tgccgttagc	aatgggcaaca	acgttgcgca	2160
aactattaac	tggcgaacta	cttactctag	cttcccggca	acaattaata	gactggatgg	2220

aggcggataa	agttgcagga	ccacttctgc	gctcggccct	tccggctggc	tggtttattg	2280
ctgataaatc	tggagccggt	gagcgtgggt	ctcgcggtat	cattgcagca	ctggggccag	2340
atggtaagcc	ctcccgtatc	gtagttatct	acacgacggg	gagtcaggca	actatggatg	2400
aacgaaatag	acagatcgct	gagatagggt	cctcactgat	taagcattgg	taactgtcag	2460
accaagttta	ctcatatata	cttttagattg	atttaaaact	tcatttttaa	tttaaaagga	2520
tctaggtgaa	gatacctttt	gataatctca	tgaccaaact	cccttaacgt	gagttttcgt	2580
tccactgagc	gtcagacccc	gtagaaaaga	tcaaaggatc	ttcttgagat	cctttttttc	2640
tgcgcgtaat	ctgctgcttg	caaacaaaaa	aaccaccgct	accagcgggt	gtttgtttgc	2700
cggatcaaga	gctaccaact	ctttttccga	aggtaactgg	cttcagcaga	gcgcagatac	2760
caaatactgt	ccttctagt	tagccgtagt	taggccacca	cttcaagaac	tctgtagcac	2820
cgctacata	cctcgctctg	ctaatacctgt	taccagtggc	tgctgccagt	ggcgataagt	2880
cgtgtcttac	cgggttggac	tcaagacgat	agttaccgga	taaggcgag	cggtcgggct	2940
gaacgggggg	ttcgtgcaca	cagcccgact	tgagcggaac	gacctacacc	gaactgagat	3000
acctacagcg	tgagctatga	gaaagcgcca	cgcttccga	agggagaaag	gcggacaggt	3060
atccggtaag	cggcagggtc	ggaacaggag	agcgacagag	ggagcttcca	gggggaaacg	3120
cctggtatct	ttatagtctt	gtcgggtttc	gccacctctg	acttgagcgt	cgatttttgt	3180
gatgctcgtc	aggggggagg	agcctatgga	aaaacgccag	caacgcggcc	tttttacggt	3240
tcctggcctt	ttgctggcct	tttgctcaca	tgcttcttcc	tgcggttatcc	cctgattctg	3300
tggataaccg	tattaccgcc	tttgagttag	ctgataccgc	tcgccgcagc	cgaacgaccg	3360
agcgcagcga	gtcagttagc	gaggaagcgg	aagagcgctt	gatgcggtat	tttctcctta	3420
cgcactgtgt	cggatattca	caccgcatat	gggtgactct	cagtacaatc	tgctctgatg	3480
ccgcatagtt	aagccagtat	acactcgctt	atcgctacag	atccggaaca	taatggtgca	3540
gggcgctgac	ttccgcgttt	ccagacttta	cgaaacacgg	aaaccgaaga	ccattcatgt	3600
tggtgtcag	gtcgcagacg	ttttgcagca	gcagtcgctt	cacgttcgct	cgcgatcggg	3660
tgattcattc	tgctaaccag	taaggcaacc	ccgccagcct	agccgggtcc	tcaacgacag	3720
gagcacgata	atgcgcaccc	gtggccagga	cccaacgctg	cccagatgac	gccgcgtgag	3780
gctgctggag	atggcgggac	cgatggatat	gttctgccaa	gggttggttt	gcgcattcac	3840
agttctccgc	aagaattgat	tggctccaat	tcttgaggtg	gtgaatccgt	tagcgagggtg	3900
ccgcgggctt	ccattcagggt	cgaggtggcc	cggctccatg	caccgcgacg	caacgcgggg	3960
aggcagacaa	ggtagagggc	ggcgccctaca	atccatgcc	acccgttcca	tggtctcgcc	4020
gaggcggcat	aaatcgccgt	gacgatcagc	gggtccagtga	tcgaagttag	gctggttaaga	4080
gccgcgagcg	atccttgaag	ctgtccctga	tggtcgctcat	ctacctgcct	ggacagcatg	4140
gcctgcaacg	cgggcatccc	gatgccgccc	gaagcgagaa	gaatcataat	ggggaaggcc	4200
atccagcctc	gcgtcgcgaa	cgccagcaag	acgtagccca	gcgcgtcggc	cgccatgccg	4260
gcgataatgg	cctgcttctc	gccgaaacgt	ttggtggcgg	gaccagttag	gaaggcttga	4320
gcgagggcgt	gcaagattcc	gaataccgca	agcgacaggc	cgatcatcgt	cgcgctccag	4380
cgaagcggt	cctcgccgaa	aatgacccag	agcgctgccc	gcacctgtcc	tacgagttgc	4440
atgataaaga	agacagtcat	aagtgcggcg	acgatagtca	tgccccgcgc	ccaccggaag	4500
gagctgactg	ggttgaaggc	tctcaagggc	atcggtcgac	gctctccctt	atgcgactcc	4560
tgcattagga	agcagcccag	tagtaggttg	aggccgttga	gcaccgccgc	cgcaaggaaat	4620
ggtgcatgca	aggagatggc	gcccacacgt	cccccgccca	cggggcctgc	caccataccc	4680
acgccgaaac	aagcgctcat	gagcccgaag	tggcgagccc	gatcttcccc	atcggtgatg	4740
tcggcgatat	aggcgccagc	aaccgcacct	gtggcgccgg	tgatgccggc	cacgatgcgt	4800
ccggcgtaga	ggatccacag	gacgggtgtg	gtcgccatga	tcgcgtagtc	gatagtggct	4860
ccaagtagcg	aagcgagcag	gactgggccc	cggccaaagc	ggtcggacag	tgctccgaga	4920
acgggtgccc	atagaaattg	catcaacgca	tatagcgcta	gcagcacgcc	atagtactgt	4980
cgatgctgt	cggaatggag	gatatcccgc	aagaggcccc	gcagtaccgg	cataaccaag	5040
cctatgccta	cagcatccag	ggtgacgggt	ccgaggatga	cgatgagcgc	attgttagat	5100
ttcatacacg	gtgcctgact	gcgttagcaa	tttaactgtg	ataaactacc	gcattaaagc	5160
taatcgatga	taagctgtca	aacatgagtg	atccgggctt	atcgactgca	cgggtgcacca	5220
atgcttcttg	cgtcaggcag	ccatcggaag	ctgtggtatg	gctgtgcagg	tcgtaaataca	5280
ctgcataatt	cgtgtcgctc	aaggcgcaat	cccgcttctg	ataatgtttt	ttgcgccgac	5340
atcataacgg	ttctggcaaa	tattctgaaa	tgagctgttg	acaattaatc	atcggtcgtg	5400
ataatgtgtg	gaattgtgag	cggataacaa	tttcacacag	gaaaca		5446

<210> 2

<211> 3225

<212> DNA

<213> Homo sapiens

<400> 2

aattcgtcaa	gcagcagtat	atgctgggtg	gagccacaat	cttcgcccc	caggctgccg	60
ctttcattat	gacggaagcg	gttttcatca	atcaggaaga	agctgacttc	cacaccagc	120
gaggcgcccc	agttttccag	caggctacat	ttacgttgta	gcaattggcg	ctcttcgcta	180
tcgagccagg	attgatgaca	gacccagata	tccaggtcag	aggaacaact	ttgccctacg	240
gacgaggtgc	tgcccatggt	gtatacacca	gtaattggaa	gctcaccttt	cggcggtacc	300
tgtactgaca	ttccacgata	cagttcaagc	tcgttcaggt	agtggcggtg	agtttcatca	360
ggcgtgtaaa	ggcaaagtcc	tttgggaacg	ttaccatcaa	ggtagcccgg	cattagcggg	420
tggtgatagt	gcaacaatgt	cggcagtaga	ctgtagacct	gttggaatgc	aggccccata	480
gcagcaagcg	cgcgatccac	acgcaattga	tttatggcat	ccagtctctg	tttcagagtc	540
tcaatataga	ggtacaagac	gtatcgctg	atttgcctac	cgtcatgact	gtgattccgc	600
caacatcaac	ggtaacacgc	ggcattcggt	atatttcgta	tgtcaaaggt	aaccgttacc	660
acttttcgcg	cctgggtttt	ttagtttcac	gacgaaaaaa	tggtctaaaa	cgtgatcaat	720
ttaacacctt	gctgattgac	cgtaaagaaa	gatgcgctac	atacaagtgt	agcaccgttt	780
attctctgta	aattccttat	tacaacggcg	tgaaacgcct	gtcaggatcc	actgccagac	840
ctcattttac	ggtttgcgca	ggcgtctacg	tttcaccaca	acactgacat	cactctggca	900
aggatgttag	gatggaccac	ggatgataat	gacggtaaca	agcatgttag	acaatgtttt	960
aagaattgcc	acacgccaaa	gcccacttgc	actctggcag	gcacactatg	tcaaagacaa	1020
gttgatggcg	agccatccgg	gcctggctgt	tgaactggta	ccgatgggtga	cctcgagcgg	1080
cagtaagag	gttccaactt	tcaccataat	gaaataagat	cactaccggg	cgtatttttt	1140
gagttgtcga	gattttcagg	agctaaggaa	gctaaaaatg	agaaaaaaat	cattggatat	1200
accaccgttg	atatatccca	atggcatcgt	aaagaacatt	ttgaggcatt	tcagtcagtt	1260
gctcaatgta	cctataacca	gaccgttcag	ctggatatta	cggccttttt	aaagaccgta	1320
aagaaaaata	agcacaagtt	ttatccggcc	tttattcaca	ttcttgcccc	cctgatgaat	1380
gctcatccgg	aattacgtat	ggcaatgaaa	gacggtgagc	tggtgatatg	ggatagtgtt	1440
cacccttggt	acaccgtttt	ccatgagcaa	actgaaacgt	tttcatcgct	ctggagtga	1500
taccacgacg	atttccggca	gtttctacac	atatattcgc	aagatgtggc	gtgttacggg	1560
gaaaacctgg	cctattttcc	ttaaagggtt	attgagaata	tggttttcgt	ctcagccaat	1620
ccctgggtga	gtttcaccag	ttttgattta	aacgtggcca	atatggacaa	cttcttcgcc	1680
cccgttttca	ccatgggcaa	atattatacg	caaggcgaca	aggtgctgat	gccgctggcg	1740
attcaggttc	atcatgccgt	ttgtgatggc	ttccatgtcg	gcagaatgct	taatgaatta	1800
caacagtact	gcgatgagtg	gcagggcggg	gcgtaattct	cgagaccggc	atgagtatcc	1860
ttgtcaccgg	cccgtctccc	gctggagaag	agttagttag	ccgtctgcgc	acactggggc	1920
aggtggcctg	gcattttccg	ctgattgagt	tttctccggg	tcaacaatta	ccgcaacttg	1980
ctgatcaact	ggcagcgctg	ggggagagcg	atctgttgtt	tgccctctcg	caacacgcgg	2040
ttgcttttgc	ccaatcacag	ctgcatcagc	aagatcgtaa	atggccccga	ctacctgatt	2100
atttcgcat	ttgacgcacc	accgcactgg	cactacatac	cgtaagtggg	cagaagattc	2160
tctaccgcga	ggatcgggaa	atcagcgaag	cttctgtaca	attacctgaa	ttacaaaaata	2220
ttgcgggcaa	acgtgcgctg	atattacgtg	gcaatgggtg	tcgtgagcta	attgggggata	2280
ccctgacggc	gcgcggtgct	gaggtcactt	tttgtgaatg	ttatcaacga	tgcgcaatcc	2340
attacgatgg	tgagaagaa	gcgatgcgct	ggcaagcccc	cgaggtgacg	atggctcgtg	2400
ttaccagcgg	tgaaatgttg	cagcaactct	ggtcgctgat	cccacaatgg	tatcgtgagc	2460
actggttact	acactgtcga	ctattggctg	tcagtgagcg	tttggcgaaa	ctcgcccggg	2520
aactgggctg	gcaagacatt	aaggtcgccg	ataacgctga	caacgatgcg	cttttacggg	2580
cattacaata	actctcataa	caggaagcca	taatgacgga	acaagaaaaa	acctccgccc	2640
tggttgagag	gaccagggag	gccgtggaca	ccagctcaca	acctgtcgca	acagaaaaaa	2700
agagtgaaga	caataccgca	ttgattctca	gcgcggtggc	tatcgctatt	gctctggcgg	2760
cgggcatcgg	tttgtatggc	tggggtaaac	aacagggcgt	caatcagacc	gccaccagcg	2820
atgccttggc	taaccaactg	acggcatttg	aaaaagccca	ggagagccaa	aaagccgagc	2880
tggaaggcat	tattaagcaa	caagctgcac	aacttaagca	ggcgaatcgt	cagcaagaaa	2940
cgctggcaaa	acagttggat	gaagtccaac	aaaaggctcg	caccatttcc	ggcagcgatg	3000
ctaaaacctg	gctgctggct	caggccgatt	ttctggtgaa	actcgccgga	cggaagctgt	3060
ggagcgatca	ggacgtcacg	accgctgcag	cgttgctgaa	aagtgcagac	gccagcctgg	3120
cggatatgaa	tgacccgagt	ctgattaccg	ttcgtcgggc	aattaccgat	gatatcgcca	3180
gcctttctgc	agtatcgag	gtggattatg	acggcatcat	cctta		3225

<210> 3
 <211> 1035
 <212> DNA
 <213> Homo sapiens

<400> 3

atgagagtga	ttcgcgtggg	taccgcaag	agccagcttg	ctcgcataca	gacggacagt	60
gtggtggcaa	cattgaaagc	ctcgtaacct	ggcctgcagt	ttgaaatcat	tgctatgtcc	120
accacagggg	acaagattct	tgatactgca	ctctctaaga	ttggagagaa	aagcctgttt	180
accaaggagc	ttgaacatgc	cctggagaag	aatgaagtgg	acctggttgt	tactccttg	240
aaggacctgc	ccactgtgct	tctcctggc	ttcaccatcg	gagccatctg	caagcgggaa	300
aaccctcatg	atgctgttgt	ctttcaccca	aaatttggtg	ggaagaccct	agaaaccctg	360
ccagagaaga	gtgtggtggg	aaccagctcc	ctgcgaagag	cagcccagct	gcagagaaa	420
ttcccgcatc	tggagttcag	gagtattcgg	ggaaacctca	acaccggct	tcggaagctg	480
gacgagcagc	aggagttcag	tgccatcatc	ctggcaacag	ctggcctgca	gcgcatgggc	540
tggcacaacc	gggttgggca	gatcctgcac	cctgaggaa	gcatgtatgc	tgtgggcccag	600
ggggccttgg	gcgtggaagt	gcgagccaag	gaccaggaca	tcttggtatc	ggtgggtgtg	660
ctgcacgatc	ccgagactct	gcttcgctgc	atcgctgaaa	gggccttcct	gaggcacctg	720
gaaggaggct	gcagtgtgcc	agtagccgtg	catacagcta	tgaaggatgg	gcaactgtac	780
ctgactggag	gagtcctggag	tctagacggc	tcagatagca	tacaagagac	catgcaggct	840
accatccatg	tccctgcca	gcataagat	ggccctgagg	atgaccaca	gttggtaggc	900
atcactgctc	gtaacattcc	acgagggcc	cagttggctg	cccagaactt	gggcatcagc	960
ctggccaact	tgttgctgag	caaaggagcc	aaaaacatcc	tggatgttgc	acggcaattg	1020
aacgatgccc	attaa					1035

<210> 4
 <211> 1113
 <212> DNA
 <213> Homo sapiens

<400> 4

cacacagcct	actttccaag	cggagccatg	tctggtaacg	gcaatgcggc	tgcaacggcg	60
gaagaaaaca	gccccaaagat	gagagtgatt	cgcgtgggta	cccgaagag	ccagcttgct	120
cgcatacaga	cggacagtgt	ggtggcaaca	ttgaaagcct	cgtaccctgg	cctgcagttt	180
gaaatcattg	ctatgtccac	cacaggggac	aagattcttg	atactgcact	ctctaagatt	240
ggagagaaaa	gcctgtttac	caaggagctt	gaacatgcc	tggagaagaa	tgaagtggac	300
ctggttggtc	actccttgaa	ggacctgccc	actgtgcttc	ctcctggctt	caccatcgga	360
gccatctgca	agcgggaaaa	ccctcatgat	gctgttgtct	ttcacccaaa	atttggtggg	420
aagaccctag	aaaccctgcc	agagaagagt	gtggtgggaa	ccagctccct	gcgaagagca	480
gcccagctgc	agagaaagt	cccgcatctg	gagttcagga	gtattcgggg	aaacctcaac	540
acccggcttc	ggaagctgga	cgagcagcag	gagttcagtg	ccatcatcct	ggcaacagct	600
ggcctgcagc	gcatgggctg	gcacaaccgg	gttgggcaga	tctgcaccc	tgaggaaatgc	660
atgtatgctg	tgggcccagg	ggccttgggc	gtggaagtgc	gagccaagga	ccaggacatc	720
ttggatctgg	tgggtgtgct	gcacgatccc	gagactctgc	ttcgctgcat	cgctgaaagg	780
gccttcctga	ggcacctgga	aggaggctgc	agtgtgccag	tagccgtgca	tacagctatg	840
aaggatgggc	aactgtacct	gactggagga	gtctggagtc	tagacggctc	agatagcata	900
caagagacca	tgcaggctac	catccatgtc	cctgccacgc	atgaagatgg	ccctgaggat	960
gacccacagt	tggtaggcat	cactgctcgt	aacattccac	gagggcccca	gttggtgctcc	1020
cagaacttgg	gcatacgcct	ggccaacttg	ttgctgagca	aaggagccaa	aaacatcctg	1080
gatgttgac	ggcaattgaa	cgatgcccac	taa			1113

<210> 5
 <211> 1035
 <212> DNA
 <213> Homo sapiens

<400> 5

atgagagtga	ttcgcgtggg	taccgcaag	agccagcttg	ctcgcataca	gacggacagt	60
gtggtggcaa	cattgaaagc	ctcgtaacct	ggcctgcagt	ttgaaatcat	tgctatgtcc	120

accacagggg	acaagattct	tgatactgca	ctctctaaga	ttggagagaa	aagcctgttt	180
accaaggagc	ttgaacatgc	cctggagaag	aatgaagtgg	acctggttgt	tcactccttg	240
aaggacctgc	ccactgtgct	tcctcctggc	ttcaccatcg	gagccatctg	caagcgggaa	300
aaccctcatg	atgctgttgt	ctttcaccca	aaatttggtg	ggaagaccct	agaaaccctg	360
ccagagaaga	gtgtgggtggg	aaccagctcc	ctgcgaagag	cagcccagct	gcagagaaag	420
ttcccgcac	tggagttcag	gagtattcgg	ggaaacctca	acacccggct	tcggaagctg	480
gacgagcagc	aggagttcag	tgccatcatc	ctggcaacag	ctggcctgca	gcgcattggc	540
tggcacaacc	gggtggggca	gatcctgcac	cctgaggaat	gcatgtatgc	tgtggggccag	600
ggggccttgg	gcgtggaagt	gcgagccaag	gaccaggaca	tcttggatct	ggtgggtgtg	660
ctgcacgac	ccgagactct	gcttcgctgc	atcgctgaaa	gggccttcct	gaggcacctg	720
gaaggaggct	gcagtgtgcc	agtagccgtg	catacagcta	tgaaggatgg	gcaactgtac	780
ctgactggag	gagtcctggg	tctagacggc	tcagatagca	tacaagagac	catgcaggct	840
accatccatg	tccttgccca	gcatgaagat	ggccctgagg	atgaccaca	gttggtaggc	900
atcactgctc	gtaacattcc	acgagggccc	cagttggctg	cccagaactt	gggcatcagc	960
ctggccaact	tgttgctgag	caaaggagcc	aaaaacatcc	tggatgttgc	acggcaattg	1020
aacgatgccc	attaa					1035

<210> 6

<211> 1035

<212> DNA

<213> Homo sapiens

<400> 6

atgagagtga	ttcgcgtggg	taccgcgaag	agccagcttg	ctcgcataca	gacggacagt	60
gtggtggcaa	cattgaaagc	ctcgtaccct	ggcctgcagt	ttgaaatcat	tgctatgtcc	120
accacagggg	acaagattct	tgatactgca	ctctctaaga	ttggagagaa	aagcctgttt	180
accaaggagc	ttgaacatgc	cctggagaag	aatgaagtgg	acctggttgt	tcactccttg	240
aaggacctgc	ccactgtgct	tcctcctggc	ttcaccatcg	gagccatctg	caagcgggaa	300
aaccctcatg	atgctgttgt	ctttcaccca	aaatttggtg	ggaagaccct	agaaaccctg	360
ccagagaaga	gtgtgggtggg	aaccagctcc	ctgcgaagag	cagcccagct	gcagagaaag	420
ttcccgcac	tggagttcag	gagtattcgg	ggaaacctca	acacccggct	tcggaagctg	480
gacgagcagc	aggagttcag	tgccatcatc	ctggcaacag	ctggcctgca	gcgcattggc	540
tggcacaacc	gggtggggca	gatcctgcac	cctgaggaat	gcatgtatgc	tgtggggccag	600
ggggccttgg	gcgtggaagt	gcgagccaag	gaccaggaca	tcttggatct	ggtgggtgtg	660
ctgcacgac	ccgagactct	gcttcgctgc	atcgctgaaa	gggccttcct	gaggcacctg	720
gaaggaggct	gcagtgtgcc	agtagccgtg	catacagcta	tgaaggatgg	gcaactgtac	780
ctgactggag	gagtcctggg	tctagacggc	tcagatagca	tacaagagac	catgcaggct	840
accatccatg	tccttgccca	gcatgaagat	ggccctgagg	atgaccaca	gttggtaggc	900
atcactgctc	gtaacattcc	acgagggccc	cagttggctg	cccagaactt	gggcatcagc	960
ctggccaact	tgttgctgag	caaaggagcc	aaaaacatcc	tggatgttgc	acggcaattg	1020
aacgatgccc	attaa					1035

<210> 7

<211> 1034

<212> DNA

<213> Homo sapiens

<400> 7

atgagagtga	ttcgcgtggg	taccgcgaag	agccagcttg	ctcgcataca	gacggacagt	60
gtggtggcaa	cattgaaagc	ctcgtaccct	ggcctgcagt	ttgaaatcat	tgctatgtcc	120
accacagggg	acaagattct	tgatactgca	ctctctaaga	ttggagagaa	aagcctgttt	180
accaaggagc	ttgaacatgc	cctggagaag	aatgaagtgg	acctggttgt	tcactccttg	240
aaggacctgc	ccactgtgct	tcctcctggc	ttcaccatcg	gagccatctg	caagcgggaa	300
aaccctcatg	atgctgttgt	ctttcaccca	aatttggttg	gaagacccta	gaaaccctgc	360
cagagaagag	tgtgggtggg	accagctccc	tgcgaagagc	agcccagctg	cagagaaagt	420
ttcccgcac	tgagttcagg	agatttcggg	gaaacctcaa	cacccggctt	cggagctggg	480
acgagcagca	ggagttcagt	gccatcatcc	tggcaacagc	tggcctgcag	cgcattggct	540
ggcacaaccg	gggtggggcag	atcctgcacc	ctgaggaatg	catgtatgct	gtggggccag	600
gggccttggg	cgtggaagtg	cgagccaagg	accaggacat	cttggatctg	gtgggtgtgc	660

tgcacgatcc	cgagactctg	cttcgctgca	tcgctgaaag	ggccttcctg	aggcacctgg	720
aaggaggctg	cagtgtgcca	gtagccgtgc	atacagctat	gaaggatggg	caactgtacc	780
tgactggagg	agtctggagt	ctagacggct	cagatagcat	acaagagacc	atgcaggcta	840
ccatccatgt	ccctgcccag	catgaagatg	gccctgagga	tgaccacacag	ttggtaggca	900
tcactgctcg	taacattcca	cgagggcccc	agttggctgc	ccagaacttg	ggcatcagcc	960
tggccaactt	gttgctgagc	aaaggagcca	aaaacatcct	ggatgttgca	cggcaattga	1020
acgatgcccc	ttaa					1034

<210> 8
 <211> 1035
 <212> DNA
 <213> Homo sapiens

<400> 8						
atgagagtga	ttcgcgtggg	tacccgcaag	agccagcttg	ctcgcataca	gacgggcagt	60
gtggtggcaa	cattgaaagc	ctcgtaccct	ggcctgcagt	ttgaaatcat	tgctatgtcc	120
accacagggg	acaagattct	tgatactgca	ctctctaaga	ttggagagaa	aagcctgttt	180
accaaggagc	ttgaacatgc	cctggagaag	aatgaagtgg	acctggttgt	tcactccttg	240
aaggacctgc	ccactgtgct	tcctcctggc	ttcaccatcg	gagccatctg	caagcgggaa	300
aaccctcatg	atgctgttgt	ctttcaccca	aaatttggtg	ggaagaccct	agaaaccctg	360
ccagagaaga	gtgtggtggg	aaccagctcc	ctgcgaagag	cagcccagct	gcagagaagg	420
ttcccgcacg	tggagttcag	gagtattcgg	ggaaacctca	acaccgggct	tcggaagctg	480
gacgagcagc	aggagttcag	tgcatcatc	ctggcaacag	ctggcctgca	gcgcatgggc	540
tggcacaacc	gggttgggca	gatcctgcac	cctgaggaat	gcatgtatgc	tgtgggccag	600
ggggccttgg	gcgtggaagt	gcgagccaag	gaccaggaca	tcttggatct	ggtgggtgtg	660
ctgcacgatc	ccgagactct	gcttcgctgc	atcgctgaaa	gggccttcct	gaggcacctg	720
gaaggaggct	gcagtgtgcc	agtagccgtg	catacagcta	tgaaggatgg	gcaactgtac	780
ctgactggag	gagtctggag	tctagacggc	tcagatagca	tacaagagac	catgcaggct	840
accatccatg	tccttgcccc	gcataagat	ggccttgagg	atgaccaca	gttggtaggc	900
atcactgctc	gtaacattcc	acgagggccc	cagttggctg	cccagaactt	gggcatcagc	960
ctggccaact	tgttgctgag	caaaggagcc	aaaaacatcc	tggatgttgc	acggcaattg	1020
aacgatgccc	attaa					1035

<210> 9
 <211> 1035
 <212> DNA
 <213> Homo sapiens

<400> 9						
atgagagtga	ttcgcgtggg	tacccgcaag	agccagcttg	ctcgcataca	gacggacagt	60
gtggtggcaa	cattgaaagc	ctcgtaccct	ggcctgcagt	ttgaaatcat	tgctatgtcc	120
accacagggg	acaagattct	tgatactgca	ctctctaaga	ttggagagaa	aagcctgttt	180
accaaggagc	ttgaacatgc	cctggagaag	aatgaagtgg	acctggttgt	tcactccttg	240
aaggacctgc	ccactgtgct	tcctcctggc	ttcaccatcg	gagccatctg	caagcgggaa	300
aaccctcatg	atgctgttgt	ctttcaccca	aaatttggtg	ggaagaccct	agaaaccctg	360
ccagagaaga	gtgtggtggg	aaccagctcc	ctgcgaagag	cagcccagct	gcagagaaaag	420
ttcccgcacg	tggagttcag	gagtattcgg	ggaaacctca	acaccgggct	tcggaagctg	480
gacgagcagc	aggagttcag	tgccatcatc	ctggcaacag	ctggcctgca	gcgcatgggc	540
tggcacaacc	gggtggggca	gatcctgcac	cctgaggaat	gcatgtatgc	tgtgggccag	600
ggggccttgg	gcgtggaagt	gcgagccaag	gaccaggaca	tcttggatct	ggtgggtgtg	660
ctgcacgatc	ccgagactct	gcttcgctgc	atcgctgaaa	gggccttcct	gaggcacctg	720
gaaggagggt	gcagtgtgcc	agtagccgtg	catacagcta	tgaaggatgg	gcaactgtac	780
ctgactggag	gagtctggag	tctagacggc	tcagatagca	tacaagagac	catgcaggct	840
accatccatg	tccttgcccc	gcataagat	ggccttgagg	atgaccaca	gttggtaggc	900
atcactgctc	gtaacattcc	acgagggccc	cagttggctg	cccagaactt	gggcatcagc	960
ctggccaact	tgttgctgag	caaaggagcc	aaaaacatcc	tggatgttgc	acggcaattg	1020
aacgatgccc	attaa					1035

<210> 10

<211> 1034
 <212> DNA
 <213> Homo sapiens

<400> 10
 atgagagtga ttccgcgtggg taccgcgaag agccagcttg ctcgcataca gacggacagt 60
 gtggtggcaa cattgaaagc ctcgtagcct ggccctgcagt ttgaaatcat tgctatgtcc 120
 accacagggg acaagattct tgatactgca ctctctaaga ttggagagaa aagcctgttt 180
 accaaggagc ttgaacatgc cctggagaag aatgaagtgg acctgggtgt tcaactccttg 240
 aaggacctgc ccactgtgct tcctcctggc ttcaccatcg gagccatctg caagcgggaa 300
 aaccctcatg atgctgttgt ctttcaccca aaatttggtg ggaagaccct agaaaccctg 360
 ccagagaaga gtgtggtggg aaccagctcc ctgcgaagag cagcccagct gcagagaaaag 420
 ttcccgcatac tggagttcag gaggattcgg ggaaacctca acaccgggtc tcggaagctg 480
 gacgagcagc aggagttcag tgccatcatc ctggcaacag ctggcctgca gcgcattgggc 540
 tggcacaacc ggggtggggca gatcctgcac cctgaggaat gcatgtatgc tgtgggccag 600
 ggggccttgg gcgtggaagt gcgagccaag gaccaggaca tcttggtatc ggtgggtgtg 660
 ctgcacgac ccgagactct gcttcgctgc atcgctgaaa gggccttcct gaggcacctg 720
 gaaggaggct gcagtgtgcc agtagccgtg catacagcta tgaaggatgg gcaactgtac 780
 ctgactggag gagtctggag tctagacggc tcagatagca tacaagagac catgcaggct 840
 accatccatg tccctgcccc gcatgaagat ggccctgagg atgaccacaca gttggtaggc 900
 atcactgctc gtaacattcc acgagggccc cagttggctg cccagaactt gggcatcagc 960
 ctggccaact tgttgctgag caaaggagcc aaaaacatcc tggatgttgc acggcaatta 1020
 acgatgcccc ttaa 1034

<210> 11
 <211> 1035
 <212> DNA
 <213> Homo sapiens

<400> 11
 atgagagtga ttccgcgtggg taccgcgaag agccagcttg ctcgcataca gacggacagt 60
 gtggtggcaa cattgaaagc ctcgtagcct ggccctgcagt ttgaaatcat tgctatgtcc 120
 accacagggg acaagattct tgatactgca ctctctaaga ttggagagaa aagcctgttt 180
 accaaggagc ttgaacatgc cctggagaag aatgaagtgg acctgggtgt tcaactccttg 240
 aaggacctgc ccactgtgct tcctcctggc ttcaccatcg gagccatctg caagcgggaa 300
 aaccctcatg atgctgttgt ctttcaccca aaatttggtg ggaagaccct agaaaccctg 360
 ccagagaaga gtgtggtggg aaccagctcc ctgcgaagag cagcccagct gcagagaaaag 420
 ttcccgcatac tggagttcag gaggattcgg ggaaacctca acaccgggtc tcggaagctg 480
 gacgagcagc aggagttcag tgccatcatc ctggcaacag ctggcctgca gcgcattgggc 540
 tggcacaacc ggggtggggca gatcctgcac cctgaggaat gcatgtatgc tgtgggccag 600
 ggggccttgg gcgtggaagt gcgagccaag gaccaggaca tcttggtatc ggtgggtgtg 660
 ctgcacgac ccgagactct gcttcgctgc atcgctgaaa gggccttcct gaggcacctg 720
 gaaggaggct gcagtgtgcc agtagccgtg catacagcta tgaaggatgg gcaactgtac 780
 ctgactggag gagtctggag tctagacggc tcagatagca tacaagagac catgcaggcc 840
 accatccatg tccctaccca gcatgaagat ggccctgagg atgaccacaca gttggtaggc 900
 atcactgctc gtaacattcc acgagggccc cagttggctg cccagaactt gggcatcagc 960
 ctggccaact tgttgctgag caaaggagcc aaaaacatcc tggatgttgc acggcaattg 1020
 aacgatgccc attaa 1035

<210> 12
 <211> 3988
 <212> DNA
 <213> Homo sapiens

<400> 12
 cacctgacgc gccctgtagc ggcgcatata gcgcggcggg tgtggtggtt acgcgcagcg 60
 tgaccgctac acttgccagc gccctagcgc ccgctccttt cgtttcttc ccttcctttc 120
 tcgccacgtt cgccggcttt ccccgtaag ctctaaatcg ggggctccct ttagggttcc 180
 gatttagtgc tttacggcac ctcgacccca aaaaacttga ttagggatg ggttcacgta 240

gtgggcccac	gccctgatag	acgggttttcc	gcccttttgac	gttggagtcc	acgttctttta	300
atagtggact	cttggtccaa	actggaàcaa	cactcaaccc	tatctcggtc	tattctttttg	360
atttataagg	gattttgccc	atttcggcct	attgggttaaa	aaatgagctg	atttaacaaa	420
aatttaacgc	gaattttaac	aaaatattaa	cgcttacaat	ttccattcgc	cattcaggct	480
gcgcaactgt	tggaagggc	gatcgggtgcg	ggcctcttcg	ctattacgcc	agctggcgaa	540
agggggatgt	gctgcaaggc	gattaagttg	ggtaacgcca	gggttttccc	agtcacgacg	600
ttgtaaaacg	acggccagtg	aattgtaata	cgactcacta	tagggcgaaat	tgggtaccgg	660
gccccccctc	gaggtcgacg	gtatcgataa	gcttattaat	gggcatcggt	caattgccgt	720
gcaacatcca	ggatgttttt	ggctcctttg	ctcagcaaca	agttggccag	gctgatgccc	780
aagtctctggg	cagccaactg	gggccctcgt	ggaatgttac	gagcagtgat	gcctaccaac	840
tgtgggtcat	cctcagggcc	atcttcatgc	tgggcagggg	catggatggg	agcctgcatg	900
gtctcttgta	tgctatctga	gccgtctaga	ctccagactc	ctccagtcag	gtacagttgc	960
ccatccttca	tagctgtatg	cacggctact	ggcacactgc	agcctccttc	caggtgcctc	1020
aggaaggccc	tttcagcgat	gcagcgaagc	agagtctcgg	gatcgtgcag	cacaccacc	1080
agatccaaga	tgctctggtc	cttggtcgcg	acttccacgc	ccaaggcccc	ctggcccaca	1140
gcatacatgc	attcctcagg	gtgcaggatc	tgcccaaccc	ggttgtgcca	gcccattgcg	1200
tgaggccag	ctgttgccag	gatgatggca	ctgaactcct	gctgctcgtc	cagcttccga	1260
agccgggtgt	tgaggtttcc	ccgaatactc	ctgaactcca	gatgcgggaa	ctttctctgc	1320
agctgggctg	ctcttcgcag	ggagctgggt	cccaccacac	tcttctctgg	cagggtttct	1380
agggctcttc	caacaaatth	tgggtgaaag	acaacagcat	catgaggggt	ttcccgtctg	1440
cagatggctc	cgatggtgaa	gccaggagga	agcacagtgg	gcaggtcctt	caaggagtga	1500
acaaccaggt	ccacttcatt	cttctccagg	gcatgttcaa	gctccttggt	aaacaggctt	1560
ttctctccaa	tcttagagag	tgcatgatca	agaactttgt	cccctgtggg	ggacatagca	1620
atgatttcaa	actcagggcc	agggtagcag	gctttcaatg	ttgccaccac	actgtccgtc	1680
tgtatgcgag	caagctggct	cttgccgggt	cccacgcgaa	tcactctcat	gaattctctg	1740
agcccggggg	atccactagt	tctagagcgg	ccgccaccgc	ggtaggagct	cagcttttgt	1800
tccctttagt	gagggttaat	tctagagctg	gcgtaatcat	ggtagtagct	gtttcctgtg	1860
tgaaattggt	atccgctcac	aattccacac	aacatacgag	ccggaagcat	aaagtgtaaa	1920
gcctgggggtg	cctaattgagt	gagctaactc	acattaattg	cgttgccgct	actgcccgtt	1980
ttccagtcgg	gaaacctgtc	gtgccagctg	cattaatgaa	tccgccaacg	cgccggggaga	2040
ggcgggttgc	gtattggggc	ctcttccgct	tctcgcgtca	ctgactcgct	gcgctcggtc	2100
gttcggctgc	ggcgagcggg	atcagctcac	tcaaaggcgg	taatacgggt	atccacagaa	2160
tcaggggata	acgcaggaaa	gaacatgtga	gcaaaaaggc	agcaaaaagg	caggaaacgt	2220
aaaaaggccg	cgttgctggc	gtttttccat	aggctccgcc	cccctgacga	gcatacaaaa	2280
aatcgacgct	caagtcagag	gtggcgaaac	ccgacaggac	tataaagata	ccaggcggtt	2340
ccccctggaa	gctccctcgt	gcgctctcct	gttccgaccc	tgcgcttac	cggatacctg	2400
tccgccttcc	tcccttcggg	aagcgtggcg	ctttctcata	gctcacgctg	taggtatctc	2460
agttcgggtg	aggctcgttc	ctccaagctg	ggctgtgtgc	acgaaccccc	cgttcagccc	2520
gaccgctgcg	ccttatccgg	taactatcgt	cttgagtcca	acccggtaag	acacgactta	2580
tcgcactggg	cagcagccac	tggtaacagg	attagcagag	cgaggtagtg	aggcgggtgt	2640
acagagtctc	tgaagtgggt	gcctaactac	ggctacacta	gaaggacagt	atttggtatc	2700
tgcgctctgc	tgaagccagt	taccttcgga	aaaagagttg	gtagctcttg	atccggcaaa	2760
caaaccaccg	ctggtagcgg	tggttttttt	gtttgcaagc	agcagattac	gcgcagaaaa	2820
aaaggatctc	aagaagatcc	tttgatcttt	tctacggggg	ctgacgctca	gtggaacgaa	2880
aactcacggt	aagggattht	gggtcatgaga	ttatcaaaaa	ggatcttcac	ctagatcctt	2940
ttaaattaaa	aatgaagttt	taaatcaatc	taaagtatat	atgagtaaac	ttgggtctgac	3000
agttaccaat	gcttaatcag	tgaggcacct	atctcagcga	tctgtctatt	tcgttcaccc	3060
atagttgcct	gactccccgt	cgtgtagata	actacgatac	gggaggggct	accatctggc	3120
cccagtgctg	caatgatacc	gcgagaccga	cgctcacccg	ctccagattt	atcagcaata	3180
aaccagccag	ccggaagggc	cgagcgcaga	agtggtcctg	caactttatc	cgcctccatc	3240
cagtctatta	attgttgccg	ggaagctaga	gtaagtgtt	cgccagttaa	tagtttgccg	3300
aacgttggtg	ccattgctac	aggcatcggt	gtgtcacgct	cgtcgttttg	tatggcttca	3360
ttcagctccg	gttcccaacg	atcaaggcga	gttacatgat	cccccatggt	gtgcaaaaaa	3420
gcgggttagct	ccttcgggtc	tccgatcggt	gtcagaagta	agttggccgc	agtgttatca	3480
ctcatgggtta	tggcagcact	gcataattct	cttactgtca	tgccatccgt	aagatgcttt	3540
tctgtgactg	gtgagtactc	aaccaagtca	ttctgagaat	agtgtatgcg	gcgaccgagt	3600
tgctcttgcc	cggcgtcaat	acgggataat	accgcgccac	atagcagaac	tttaaaagtg	3660
ctcatcattg	gaaaacgttc	ttcggggcga	aaactctcaa	ggatcttacc	gctgttgaga	3720
tccagttcga	tgtaaaccac	tccgtgcaccc	aactgatctt	cagcatcttt	tactttcacc	3780

agcgtttctg	ggtgagcaaa	aacaggaagg	caaaatgccg	caaaaaaggg	aataagggcg	3840
acacggaaat	ggtgaatact	catactcttc	ctttttcaat	attattgaag	catttatcag	3900
ggttattgtc	tcattgagcg	atacatattt	gaatgtattt	agaaaaataa	acaaataggg	3960
gttcgcgcga	catttccccg	aaaagtgc				3988

<210> 13

<211> 1260

<212> DNA

<213> Homo sapiens

<400> 13

cacaggaaac	agctatgacc	atgattacgc	caagctcgaa	attaaccctc	actaaagggg	60
acaaaagctg	gagctccacc	gcggtggcgg	ccgctctaga	actagtggat	cccccgggct	120
gcaggaattc	atgagagtga	ttcgcgtggg	taccgcgaag	agccagcttg	ctcgcataca	180
gacggacagt	gtggtggcaa	cattgaaagc	ctcgtaccct	ggcctgcagt	ttgaaatcat	240
tgctatgtcc	accacagggg	acaagattct	tgatactgca	ctctctaaga	ttggagagaa	300
aagcctgttt	accaaggagc	ttgaacatgc	cctggagaag	aatgaagtgg	acctggttgt	360
tcactccttg	aaggacctgc	ccactgtgct	tcctcctggc	ttcaccatcg	gagccatctg	420
caagcgggaa	aacctcatg	atgctgttgt	ctttcaccca	aaatttggtg	ggaagaccct	480
agaaaccttg	ccagagaaga	gtgtggtggg	aaccagctcc	ctgcgaagag	cagcccagct	540
gcagagaaag	ttcccgcatc	tggagtccag	gagtattcgg	ggaaacctca	acaccgggct	600
tcggaagctg	gacgagcagc	aggagtccag	tgccatcatc	ctggcaacag	ctggcctgca	660
gcgcatgggc	tggcacaacc	gggttgggca	gatcctgcac	cctgaggaat	gcatgtatgc	720
tgtgggccag	ggggccttgg	gcgtggaagt	gcgagccaag	gaccaggaca	tcttggatct	780
ggtgggtgtg	ctgcacgatc	ccgagactct	gcttcgctgc	atcgctgaaa	gggccttcct	840
gaggcacctg	gaaggaggct	gcagtgtgcc	agtagccgtg	catacagcta	tgaaggatgg	900
gcaactgtac	ctgactggag	gagtctggag	tctagacggc	tcagatagca	tacaagagac	960
catgcaggct	accatccatg	tccttgccca	gcatgaagat	ggccctgagg	atgaccacac	1020
gttggtaggc	atcactgctc	gtaacattcc	acgagggccc	cagttaggctg	cccagaactt	1080
gggcatcagc	ctggccaact	tgttgctgag	caaaggagcc	aaaaacatcc	tggatgttgc	1140
acggcaattg	aacgatgccc	attaataagc	ttatcgatac	cgtcgacctc	gagggggggc	1200
ccggtaccca	attcgcccta	tagtgagtcg	tattacaatt	cactggccgt	cgttttacaa	1260

<210> 14

<211> 32

<212> DNA

<213> Homo sapiens

<400> 14

atccatgaat	tccacgcaat	gcagccccag	tc	32
------------	------------	------------	----	----

<210> 15

<211> 32

<212> DNA

<213> Homo sapiens

<400> 15

agtcgtaagc	ttgcctggca	ctgtcctcca	tc	32
------------	------------	------------	----	----

<210> 16

<211> 22

<212> DNA

<213> Homo sapiens

<400> 16

gtaatacgac	tcactatagg	gc	22
------------	------------	----	----

<210> 17

<211> 22

<212> DNA

<213> Homo sapiens				
<400> 17				
ctaaagggaa caaaagctgg ag				22
<210> 18				
<211> 20				
<212> DNA				
<213> Homo sapiens				
<400> 18				
gcgcgtaata cgactcacta				20
<210> 19				
<211> 20				
<212> DNA				
<213> Homo sapiens				
<400> 19				
cctacgctgt gtcttgatct				20
<210> 20				
<211> 20				
<212> DNA				
<213> Homo sapiens				
<400> 20				
ggcttcacca tgagcatgtc				20
<210> 21				
<211> 993				
<212> DNA				
<213> Homo sapiens				
<400> 21				
atgcagcccc agtccgttct gcacagcggc tacttccacc cactacttcg ggcctggcag				60
acagccacca ccaccctcaa tgcctccaac ctcatctacc ccattcttgt cacggatgtt				120
cctgatgaca tacagcctat caccagcctc ccaggagtgg ccaggatatgg tgtgaagcgg				180
ctggaagaga tgctgaggcc cttagtgga gagggcctac gctgtgtctt gatctttggc				240
gtccccagca gaggttccaa ggacgagcgg gggtccgcag ctgactccga ggagtcccca				300
gctattgagg caatccatct gttgaggaag acgttcccca acctcctggg ggcctgtgat				360
gtctgacctgt gtccctacac ctcccatggg cactgcgggc tcctgagtga aaacggagca				420
ttccgggctg aggagagccg ccagcggctg gctgaggtgg cattggcgta tgccaaggca				480
ggatgtcagg tggtagcccc gtcggacatg atggatggac gcgtggaagc catcaaagag				540
gccctgatgg cacatggact tggcaacagg gtatcgggtga tgagctacag tgccaaattt				600
gcttctgtt tctatggccc ttccgggat gcagctaagt caagcccagc ttttggggagc				660
cgccgctgct accagctgcc ccctggagca cgaggcctgg ctctccgagc tgtggaccgg				720
gatgtacggg aaggagctga catgctcatg gtgaagccgg gaatgcccta cctggacatc				780
gtgcgggagg taaaggacaa gcacctgac ctccctctcg ccgtgtacca cgtctctgga				840
gagtttgcca tgctgtggca tggagcccag gccggggcat ttgatctcaa ggctgccgta				900
ctggaggcca tgactgcctt ccgcagagca ggtgctgaca tcatcatcac ctactacaca				960
ccgcagctgc tgcagtggct gaaggaggaa tga				993
<210> 22				
<211> 330				
<212> PRT				
<213> Homo sapiens				
<400> 22				

Met	Gln	Pro	Gln	Ser	Val	Leu	His	Ser	Gly	Tyr	Phe	His	Pro	Leu	Leu		
1				5					10					15			
Arg	Ala	Trp	Gln	Thr	Ala	Thr	Thr	Thr	Leu	Asn	Ala	Ser	Asn	Leu	Ile		
			20						25				30				
Tyr	Pro	Ile	Phe	Val	Thr	Asp	Val	Pro	Asp	Asp	Ile	Gln	Pro	Ile	Thr		
		35					40					45					
Ser	Leu	Pro	Gly	Val	Ala	Arg	Tyr	Gly	Val	Lys	Arg	Leu	Glu	Glu	Met		
	50					55					60						
Leu	Arg	Pro	Leu	Val	Glu	Glu	Gly	Leu	Arg	Cys	Val	Leu	Ile	Phe	Gly		
65				70					75						80		
Val	Pro	Ser	Arg	Val	Pro	Lys	Asp	Glu	Arg	Gly	Ser	Ala	Ala	Asp	Ser		
			85					90						95			
Glu	Glu	Ser	Pro	Ala	Ile	Glu	Ala	Ile	His	Leu	Leu	Arg	Lys	Thr	Phe		
			100					105					110				
Pro	Asn	Leu	Leu	Val	Ala	Cys	Asp	Val	Cys	Leu	Cys	Pro	Tyr	Thr	Ser		
		115					120					125					
His	Gly	His	Cys	Gly	Leu	Leu	Ser	Glu	Asn	Gly	Ala	Phe	Arg	Ala	Glu		
	130				135						140						
Glu	Ser	Arg	Gln	Arg	Leu	Ala	Glu	Val	Ala	Leu	Ala	Tyr	Ala	Lys	Ala		
145				150						155					160		
Gly	Cys	Gln	Val	Val	Ala	Pro	Ser	Asp	Met	Met	Asp	Gly	Arg	Val	Glu		
			165					170						175			
Ala	Ile	Lys	Glu	Ala	Leu	Met	Ala	His	Gly	Leu	Gly	Asn	Arg	Val	Ser		
			180					185					190				
Val	Met	Ser	Tyr	Ser	Ala	Lys	Phe	Ala	Ser	Cys	Phe	Tyr	Gly	Pro	Phe		
		195					200					205					
Arg	Asp	Ala	Ala	Lys	Ser	Ser	Pro	Ala	Phe	Gly	Asp	Arg	Arg	Cys	Tyr		
	210				215						220						
Gln	Leu	Pro	Pro	Gly	Ala	Arg	Gly	Leu	Ala	Leu	Arg	Ala	Val	Asp	Arg		
225				230						235					240		
Asp	Val	Arg	Glu	Gly	Ala	Asp	Met	Leu	Met	Val	Lys	Pro	Gly	Met	Pro		
			245					250						255			
Tyr	Leu	Asp	Ile	Val	Arg	Glu	Val	Lys	Asp	Lys	His	Pro	Asp	Leu	Pro		
		260					265						270				
Leu	Ala	Val	Tyr	His	Val	Ser	Gly	Glu	Phe	Ala	Met	Leu	Trp	His	Gly		
	275						280					285					
Ala	Gln	Ala	Gly	Ala	Phe	Asp	Leu	Lys	Ala	Ala	Val	Leu	Glu	Ala	Met		
	290				295						300						
Thr	Ala	Phe	Arg	Arg	Ala	Gly	Ala	Asp	Ile	Ile	Ile	Thr	Tyr	Tyr	Thr		
305				310						315					320		
Pro	Gln	Leu	Leu	Gln	Trp	Leu	Lys	Glu	Glu								
			325						330								